

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 February 2002 (07.02.2002)

PCT

(10) International Publication Number
WO 02/09698 A1

- (51) International Patent Classification⁷: A61K 31/34 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (21) International Application Number: PCT/US01/24053
- (22) International Filing Date: 1 August 2001 (01.08.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/222,190 1 August 2000 (01.08.2000) US
09/919,510 31 July 2001 (31.07.2001) US
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Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



A1

WO 02/09698 A1
(54) Title: COMPOSITIONS EXHIBITING SYNERGISTIC INHIBITION OF THE EXPRESSION AND/OR ACTIVITY OF CYCLOOXYGENASE-2

(57) Abstract: A novel formulation is provided that serves to inhibit the inflammatory response in animals. The formulation comprises, as a first component, a diterpene triepoxide lactone species or a sesquiterpene lactone species and, as a second component, at least one member selected from the group consisting of a diterpene triepoxide lactone species, a sesquiterpene lactone species, a diterpene lactone species, and a triterpene species or derivatives thereof with the proviso that the same first component cannot also serve as the second component, and provides synergistic anti-inflammatory effects in response to physical or chemical injury or abnormal immune stimulation due to a biological agent or unknown etiology.

**COMPOSITIONS EXHIBITING SYNERGISTIC INHIBITION OF
THE EXPRESSION AND/OR ACTIVITY OF CYCLOOXYGENASE-2**

RELATED APPLICATIONS AND PRIORITY CLAIM

5 This application claims the benefit of US Provisional Application No.
60/222,190 filed August 1, 2000.

FIELD OF THE INVENTION

The present invention relates generally to a composition exhibiting synergistic inhibition of the expression and/or activity of inducible cyclooxygenase-2 (COX-2). More particularly, the composition comprises, as a first component, a diterpene triepoxide lactone species or a sesquiterpene lactone species and, as a second component, at least one member selected from the group consisting of a diterpene triepoxide lactone species, a sesquiterpene lactone species, a diterpene lactone species, and a triterpene species or derivatives thereof with the proviso that the same first component cannot also simultaneously serve as the second component. The composition functions synergistically to inhibit the inducibility and/or activity of inducible cyclooxygenase (COX-2) with no significant effect on constitutive cyclooxygenase (COX-1)

BACKGROUND OF THE INVENTION

Inflammatory diseases affect more than fifty million Americans. As a result of basic research in molecular and cellular immunology over the last ten to fifteen years, approaches to diagnosing, treating and preventing these immunologically-based diseases has been dramatically altered. One example of this is the discovery of an inducible form of the cyclooxygenase enzyme. Constitutive cyclooxygenase (COX), first purified in 1976 and cloned in 1988, functions in the synthesis of prostaglandins (Pgs) from arachidonic acid (AA).

25 Three years after its purification, an inducible enzyme with COX activity was identified and given the name COX-2, while constitutive COX was termed COX-1.

COX-2 gene expression is under the control of pro-inflammatory cytokines and growth factors. Thus, the inference is that COX-2 functions in both inflammation and control of cell growth. While COX-2 is inducible in many tissues, it is present constitutively in the brain and spinal cord, where it
5 may function in nerve transmission for pain and fever. The two isoforms of COX are nearly identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. Protective PGs , which preserve the integrity of the stomach lining and maintain normal renal function in a compromised kidney, are synthesized by COX-1. On the other
10 hand, PGs, synthesized by COX-2 in immune cells are central to the inflammatory process.

The discovery of COX-2 has made possible the design of drugs that reduce inflammation without removing the protective PGs in the stomach and kidney made by COX-1. These selective COX-2 inhibitors may not only be
15 anti-inflammatory, but may also be actively beneficial in the prevention and treatment of colon cancer and Alzheimer's disease.

An ideal formulation for the treatment of inflammation would inhibit the induction and activity of COX-2 without affecting the activity of COX-1. Historically, the non-steroidal and steroidal anti-inflammatory drugs used for
20 treatment of inflammation lack the specificity of inhibiting COX-2 without affecting COX-1. Therefore, mores anti-inflammatory drugs damage the gastrointestinal system when used for extended periods. Thus, new treatments for inflammation and inflammation-based diseases are urgently needed.

The natural pharmacopoeia of plants and herbs used in traditional medicines for treatment of inflammatory conditions was recently found to contain COX-2 inhibitors. One such plant is *Tripterygium wilfordi* (TW). This herb, known as *Lei Gong Teng* in China, has been used to treat patients suffering with rheumatoid arthritis with a 92% efficacy rate. *Lei Gong Teng* is available in the U.S. and is advertised to support the healthy functioning of bone joints
30 (www.China-Med.net).

Over 60 compounds have been isolated from TW, and many have been identified as having anti-inflammatory and immunosuppressive activity. Representative compounds that have been isolated from TW include triptolide, 16-hydroxytriptolide, triptophenolide, tripdiolide, and celastrol. However, the administration and therapeutic effectiveness of these compounds have general 5 been limited by their low margins of safety.

Triptolide is one of the active, nonalkaloid principles isolated from TW and possesses an extensive suppressive effect on immune function, especially on T and B lymphocytes. Structurally, triptolide is a member of the group of diterpene triepoxide lactones (FIG 1). The inhibitory effect is directed and believed to occur through the inhibition of interluken-2 (IL-2) production and IL-2R (receptor) expression (Tao, et al. (1995) J. Pharmacol. Exp. Therap. 272:1305; U.S. Patent 5,500,340 to Lipsky et al. March 19, 1996). Clinical trials show that it significantly inhibits the proliferation of peripheral blood 10 mononuclear cells of rheumatic arthritis patients. After receiving this medication, patients usually indicate that stiffness, walking, and hand strength are improved with a decrease in inflammation index. Although not generally life-threatening, adverse effects of triptolide are relatively common in the clinical setting. Approximately 28% of patients taking this compound show 15 some type of side effects, such as gastrointestinal disturbance, nausea and vomiting, hypotension and edema.

Therefore, while triptolide may be useful as an anti-inflammatory agent, it can be toxic even in clinically effective doses. Other researchers have used the triptolide molecule as a starting point for the synthesis of novel analogs 20 expressing similar immune effects, while exhibiting lower toxicity (U.S. Patent 5,962,516 to Qi et al. October 5, 1999). Rather than modifying the triptolide molecule to achieve greater efficacy and lower toxicity, it is an object of this invention to combine triptolide, or a representative diterpene epoxide lactone, with a second molecule to produce a synergistic effect in the target cell. One 25 such synergistic response would be the inhibition of inducible COX-2.

Leaves or infusions of feverfew, *Tanacetum parthenium*, have long been used as a folk remedy for the relief of fever, arthritis and migraine headaches. Previous reports using feverfew extracts have suggested interference with arachidonate metabolism as the mechanism behind these pharmacological effects. In one study (Sumner et al. (1992) Biochem. Pharmacol. 43:2313-2320), crude chloroform extracts of fresh feverfew leaves produced dose-dependent inhibition of the generation of thromboxane B₂ and leukotriene B₄ by ionophore- and chemoattractant-stimulated rat peritoneal leukocytes and human polymorphonuclear leukocytes. Other research has suggested inhibition of platelet aggregation and the platelet release reaction by feverfew extracts (Groenewegen et al. (1986) J. Pharm. Pharmacol. 38:709-712). Numerous publications suggest that the biologically active components of feverfew are sesquiterpene lactones, with parthenolide being the most abundant.

In the literature approximately 25, separate biological effects have been reported for parthenolide. The potential pharmacological activities range from the inhibition of isolated bovine prostaglandin synthetase (Pugh and Sambo (1988) J. Pharm. Pharmacol. 40:743-745) to the prevention of ethanol-induced gastric ulcers in the rat (Tournier et al. (1999) J. Pharm. Pharmacol. 51:215-219). Research at the molecular level has described parthenolide inhibition of nuclear factor kappa B (NF- κ B) activation in several cell-based systems (Hehner et al. (1999) J. Immunol. 163:5617-5623; Bork et al. (1997) FEBS Letters 402:85-90) and inhibition of inducible nitric oxide gene expression in cultured rat aortic smooth muscle cells (Wong and Menendez (1999) Biochem. Biophys. Res. Commun. 262:375-380). While these molecular events may account, in part, for some of the biological actions of parthenolide, there exists no consensus on the exact nature of the underlying mechanism for its anti-inflammatory effects.

Clinically effective doses of parthenolide for migraine prevention are on the order of micrograms per kg body weight daily. Human clinical trials have verified the minimum effective doses for migraine prevention, as well as the associated discomfort of nausea and vomiting associated with use of 125 mg of

feverfew extract per day. The feverfew extracts used in these trials generally contained between 0.2 to 0.7 percent parthenolide. Therefore, the minimally effective dose of parthenolide would be estimated to be approximately 250 micrograms per day or 4 micrograms parthenolide per daily dose. While more than sufficient to effectively control migraine frequency, it is doubtful that these doses of parthenolide would be sufficient to address inflammatory responses.

Research literature on the *in vitro* anti-inflammatory effects of parthenolide reports inhibitory constants in the micromolar range. Assuming a volume of distribution greater than several hundred mL per kg and a median resonance time less than 12 hours, these parthenolide concentrations could only be achieved and maintained *in vivo* with dosing mg amounts of parthenolide per kg bodyweight. While such dosing studies have been performed successfully in laboratory animals, no clinical reports describe similar doses of parthenolide in humans. Based upon these estimates, a clinically successful preparation of parthenolide for inflammatory conditions would be required to delivery at least 15 mg parthenolide/kg-day. However, such relatively high doses of parthenolide would be commercially prohibitive due to the cost of production, even for a therapeutic formulation.

Diterpene lactone species such as andrographolide, and triterpene species, such as ursolic acid and oleanolic acid, are commonly found in plants and are used for their anti-inflammatory properties. The anti-inflammatory effects of these compounds have been described in the literature since 1960. Their mechanism of action is believed to be due (i) to the inhibition of histamine release from mast cells or (ii) to the inhibition of lipoxygenase and cyclooxygenase activity thereby reducing the synthesis of inflammatory factors produced during the arachidonic acid cascade. Since andrographolide and oleanolic acid have been found to promote the healing of stomach ulcers, it is unlikely that the cyclooxygenase activity that is inhibited is COX-1. Also, andrographolide and oleanolic are potent antioxidants, capable of inhibiting the generation of reactive oxygen intermediates and restoring tissue glutathione levels following stress.

Combinations of botanicals containing triptolide, oleanolic acid along with other herbs have been used in both traditional and commercial medicine. However, the triptolide content of TW is only 0.1%, leaving 99.9% of the ingredients of TW as undefined. Thus, it would be useful to identify a compound that would specifically enhance the anti-inflammatory effect of triptolide so that it could be used at sufficiently low doses or at current clinical doses with no adverse side effects. The optimal formulation of triptolide for preserving the health of joint tissues, for treating arthritis or other inflammatory conditions has not yet been discovered. A formulation combining triptolide and parthenolide to synergistically inhibit COX-2 and support the normalization of joint function has not yet been described.

While glucosamine is generally accepted as being effective and safe for treating osteoarthritis, medical intervention into the treatment of degenerative joint diseases is generally restricted to the alleviation of its acute symptoms. Medical doctors generally utilize non-steroidal and steroid anti-inflammatory drugs for treatment of osteoarthritis. These drugs, however, are not well-adapted for long-term therapy because they not only lack the ability to promote and protect cartilage, they can actually lead to degeneration of cartilage or reduction of its synthesis. Moreover, most non-steroidal, anti-inflammatory drugs damage the gastrointestinal system when used for extended periods. Thus, new treatments for arthritis are urgently needed.

The joint-protective properties of glucosamine would make it an attractive therapeutic agent for osteoarthritis except for two drawbacks: (i) the rate of response to glucosamine treatment is slower than for treatment with anti-inflammatory drugs, and (ii) glucosamine may fail to fulfill the expectation of degenerative remission. In studies comparing glucosamine with non-steroidal anti-inflammatory agents, for example, a double-blinded study comparing 1500 mg glucosamine sulfate per day with 1200 mg ibuprofen, demonstrated that pain scores decreased faster during the first two weeks in the ibuprofen patients than in the glucosamine-treated patients. However, the reduction in pain scores continued throughout the trial period in patients receiving glucosamine and the

difference between the two groups turned significantly in favor of glucosamine by week eight. Lopes Vaz, A., *Double blind clinical evaluation of the relative efficacy of ibuprofen and glucosamine sulphate in the management of osteoarthritis of the knee in outpatients*, 8 Curr. Med Res Opin. 145-149 (1982).
5 Thus, glucosamine may relieve the pain and inflammation of arthritis at a slower rate than the available anti-inflammatory drugs.

An ideal formulation for the normalization of cartilage metabolism or treatment of osteoarthritis would provide adequate chondroprotection with potent anti-inflammatory activity. The optimal dietary supplement for
10 osteoarthritis should enhance the general joint rebuilding qualities offered by glucosamine and attenuate the inflammatory response without introducing any harmful side effects. It should be inexpensively manufactured and comply with all governmental regulations.

However, the currently available glucosamine formulations have not
15 been formulated to optimally attack and alleviate the underlying causes of osteoarthritis and rheumatoid arthritis. Moreover, as with many commercially-available herbal and dietary supplements, the available formulations do not have a history of usage, nor controlled clinical testing, which might ensure their safety and efficacy.

20 It would be useful to identify a compound that would specifically and synergistically enhance the anti-inflammatory effect of parthenolide and/or triptolide so that these could be used at sufficiently low doses or at current clinical doses with no adverse side effects.

SUMMARY OF THE INVENTION

25 The present invention provides a composition comprising, as a first component, a diterpene triepoxide lactone species or a sesquiterpene lactone species and a second compound that specifically and synergistically enhances the anti-inflammatory effect of the first component diterpene triepoxide lactone species or the sesquiterpene lactone species. To clarify, there must be either a
30 diterpene triepoxide lactone species or a sesquiterpene lactone species as a first component. The second component can be any species selected from the group

consisting of a diterpene lactone species and a triterpene species or derivatives thereof with the proviso that the second component must be different from the first component species. In other words, the first species can be either a diterpene triepoxide lactone species or a sesquiterpene lactone species combined
5 with one or more species selected from the group consisting of a diterpene triepoxide lactone species, a sesquiterpene lactone species, a diterpene lactone species, and a triterpene species or derivatives or mixtures thereof. For example, (a) the first component can be a diterpene triepoxide lactone species and the second component can be a different diterpene triepoxide lactone species or, the first component can be a sesquiterpene lactone species and the second component can be a different sesquiterpene lactone species; (b) the first component can be a diterpene triepoxide lactone species and the second component can be a sesquiterpene lactone species; (c) the first component can be a diterpene triepoxide lactone species or a sesquiterpene lactone species and the second component can be a diterpene lactone species or a triterpene species.
10 Any diterpene triepoxide lactone, sesquiterpene lactone, diterpene lactone or triterpene species is inclusive of derivatives of the respective genus. The composition of the present invention must contain, at a minimum, two species one each representing the first component and the second component. However,
15 additional species or mixtures of species within the various genera may be present in the composition which is limited in scope only by the combinations of species within the various genera that exhibit the claimed synergistic functionality.

The composition functions synergistically to inhibit the inducibility
20 and/or activity of inducible COX-2 with little or no effect on COX-1.

The present invention further provides a compositions of matter which enhances the function of glucosamine or chondroitin sulfate to normalize joint movement or reduce the symptoms of osteoarthritis.

One specific embodiment of the present invention is a composition
30 comprising an effective amount of triptolide and at least one compound selected

from the group consisting of parthenolide, andrographilide, ursolic acid and oleanolic acid.

Another specific embodiment of the present invention is a composition comprising an effective amount of parthenolide and at least one compound selected from the group consisting triptolide, andrographolide, ursolic acid and oleanolic acid.

The present invention further provides a method of dietary supplementation and a method of treating inflammation or inflammation-based diseases in a warm-blooded animal which comprises providing to the animal suffering symptoms of inflammation the composition of the present invention containing a second component which specifically and synergistically enhances the anti-inflammatory effect of a diterpene triepoxide lactone, or a sesquiterpene lactone, and continuing to administer such a dietary supplementation of the composition until said symptoms are eliminated or reduced.

15

BRIEF DESCRIPTIONS OF THE DRAWINGS

FIG. 1 illustrates the general chemical structure of [A1] the diterpene triepoxide lactone genus and [A2] triptolide as a species within that genus.

20 FIG. 2, [A1] and [A2] respectively, illustrate the general chemical structures of the sesquiterpene lactone genus and parthenolide as a species within that genus.

FIG. 2 [B1] and [B2] respectively illustrate the general chemical structures of the diterpene lactone genus and andrographolide as a species within that genus.

25 FIG. 2 [C1], [C2] and [C3] respectively, illustrate the general chemical structures of the triterpene genus and ursolic acid and oleanolic acid as species within that genus.

FIG. 3 provides a schematic for the experimental design of EXAMPLE 1.

30 FIG. 4(a)-(g) are line graphs depicting the percent inhibition of COX-2 enzyme protein expression by individual and the combinations of the tested

materials, as described in EXAMPLE 1-7, in the absence and presence of arachidonic acid (AA).

DETAILED DESCRIPTION OF THE INVENTION

5 Before the present composition and methods of making and using thereof are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, as process steps, and materials may vary somewhat. It is also intended to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is
10 not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly directs otherwise.

15 The present invention provides a composition having a synergistic inhibitory effect on the expression and/or activity of COX-2. More particularly, the composition comprises, as a first component, an active diterpene triepoxide lactone or an active sesquiterpene lactone and, as a second component, at least one member selected from the group consisting of diterpene triepoxide lactones, active sesquiterpene lactones, diterpene lactones, and triterpenes or derivatives thereof as more specifically described above. Preferably, the molar ratio of the active first component, i.e. a diterpene triepoxide lactone species or a sesquiterpene lactone species to a second component, i.e. the member selected from the group consisting of a diterpene triepoxide lactone species, a
20 sesquiterpene lactone species, a diterpene lactone species, and a triterpene species or derivatives thereof is within a range of 1:1 to 1:10, and more preferably within a range of 1:2.5 to 1:10. The composition provided by the present invention can be formulated as a dietary supplement or therapeutic composition. The composition functions synergistically to inhibit the
25 inducibility and/or activity of COX-2 with little or no effect on COX-1.
30

As used herein, the term "dietary supplement" refers to compositions consumed to affect structural or functional changes in physiology. The term "therapeutic composition" refers to any compounds administered to treat or prevent a disease.

5 As used herein, the term "active diterpene triepoxide lactone" or "active sesquiterpene lactone" refers to a species within the diterpene triepoxide lactone or sesquiterpene lactone genera that is capable of inhibiting the inducibility and/or activity of COX-2 while having little or no effect on COX-1 or is capable of inhibiting or reducing the severity of a severe inflammatory response. All
10 active sesquiterpene lactone species have an α -methylene or γ -lactone functional group and are capable of inhibiting or reducing the severity of an inflammatory response.

15 As used herein, diterpene triepoxide lactones, diterpene lactones, sesquiterpenes lactones, triterpenes or derivatives thereof refers to naturally occurring or synthetic derivatives of species within the scope of the respective general. Representative species within each genus are listed in Table 1. Of the species listed under each genus in Table 1, those containing at least one asterisk (*) are preferred and those containing two asterisks (**) are particularly preferred.

Table 1

DITERPENE TRIPOSIDES LACTONES	DITERPENE LACTONES	ACTIVE SESQUITERPENE LACTONES	TRITERPENES
Tripchlorolide*	Andrographolide**	5- α -Hydroxy-dehydrocostuslacone	18- α -Glycyrrhetic acid**
5 Tripdiolide*	Edelin lactone	Burrodin*	18- β -Glycyrrhetic acid**
Triptolide**	Selenoandrographolide*	Chlorochrymorin	2- α ,3- α -Dihydroxyurs-12-3n-28-oic acid*
10 Tritonide**	Deoxyandrographolide**	Chrysandiol	2- α -Hydroxyursolic acid*
	Neoandrographolide**	Chrysartemin A	30Oxo-ursolic acid*
	Homoandrographolide*	Chrysartemin B	Betulin**
10 Androphan*	Cinererin	Betulinic acid**	
	Confertiflorin*	Celastrin*	
	Andrographosterin*	Costunolide*	Eburicoic acid
	14-deoxy-11-Oxoandrographolide**	Curcolone	Friedelin*
	14-deoxy-11, 12-Didehydroandrographolide**	Cynaropcrin	Glycyrrhizin
15 Andrographiside*	Dehydrocostuc lactone	Gypsogenin	
	Dehydrozaluzanin C	Oleanolic acid**	
	Deoxylactucin	Oleanolic acid-3-acetate	
	Encelin**	Pachymic acid	
20 Enhydrin**	Sophoradiol		
	Eremanthin	Soyasapogenol A	
	Eupaformonin	Soyasapogenol B	
	Eupaformosanin	Triterpin**	
	Eupatolide	Tropiophenolide*	
	Furanodienone	Tumulosic acid	
25 Heleenalin*		Ursolic acid**	
	Heterogorgiolide	Ursolic acid-3-acetate	
	Lactucin	Uvaol*	
	Leucanthin B**	-Sitosterol	
	Magnoliade		
30 Melapordin A**			
	Michelenolide		
	Parthenolide**		
	Psilotachyn A*		
	Repin		
35 Spirafolide			
	Strigol*		
	Tenulin*		
	Zaluzanin C		

"Conjugates" of diterpene triepoxide lactones, diterpene lactones, sesquiterpenes lactones, triterpenes or derivatives thereof means diterpene triepoxide lactones, diterpene lactones, sesquiterpenes lactones, triterpenes covalently bound or conjugated to a member selected from the group consisting of mono- or di- saccharides, amino acids, sulfates, succinate, acetate and glutathathione. Preferably, the mono- or di- saccharides is a member selected from the group consisting of glucose, mannose, ribose, galactose, rhamnose, arabinose, maltose, and fructose.

Therefore, one preferred embodiment of the present invention is a composition comprising a combination of an effective amount of parthenolide or triptolide, as a first component, and a second compound selected from the group consisting of triptolide, parthenolide, andrographolide, urollic acid and oleanolic acid with the proviso that there must be a combination and the first and second component cannot be the same compound, e.g. cannot be the same species within the same genus. The resulting formulation of these combinations functions to synergistically inhibit the inducibility and/or activity of COX-2 while showing little or no effect on COX-1. Therefore, the composition of the present invention essentially eliminates the inflammatory response rapidly without introducing any harmful side effects.

Preferably, the diterpene triepoxide lactone or triptolide (FIG.1 [A1] and [A2]) employed in the present invention is a pharmaceutical grade botanical extract such as can be obtained commercially, for example, from Folexco Flavor Ingredients, 150 Domorah Drive, Montogomeryville PA 18936. The triptolide used can be readily obtained from *Tripterygium wilfordii*. Pharmaceutical grade triptolide is standardized to have a triptolide content of greater than 50 percent. Additionally, it contains no alkaloids or glycosides normally found with the triptolide generally isolated from botanical sources. The pharmaceutical, botanical grade extract must pass extensive safety and efficacy procedures. As employed in the practice of the present invention, the botanical extract has a triptolide content of about 1 to 50 percent by weight. Preferably, the minimum

triptolide content is about 1 percent by weight. Alternatively, the triptolide may be synthesized using standard techniques known in chemical synthesis.

The sesquiterpene lactone genus, as represented by Fig. 2, [A1], and specifically the species parthenolide as represented by fig. 2 [A2] is preferably a pharmaceutical grade preparation such as can be obtained from Folexco Flavor Ingredients, 150 Domorah Drive, Montogomeryville, PA 18936.

5 *Chrysanthemum parthenium* or *Tanacetum vulgare* serve as ready sources of parthenolide. The pharmaceutical grade extract must pass extensive safety and efficacy procedures. Pharmaceutical grade parthenolide extract is greater than 5
10 weight percent. As employed in the practice of the invention, the extract has a parthenolide content of about 5 to 95 percent by weight. Preferably, the minimum parthenolide content is greater than 50 percent by weight. Without limiting the invention, it is anticipated that parthenolide would act to prevent an increase in the rate of transcription of the COX-2 gene by the transcriptional
15 regulatory factor NF-kappa B.

The essence of the present invention is that, rather than modifying the diterpene triepoxide lactone or active sesquiterpene lactone molecules to achieve greater efficacy and lower toxicity, a second component is added that acts in a synergistic manner. Therefore, this invention relates to the discovery
20 that when combining diterpene triepoxide lactone or active sesquiterpene lactone with a second molecule selected from the group consisting of dieterpene triepoxide lactone, sesquiterpene lactone, diterpene lactone, and triterpene or derivatives thereof, the combination produces a synergistic effect in the target cell. One such synergistic response would be the specific inhibition of inducible
25 COX-2. Preferably, the second molecule is a member selected from the group consisting of triptolide, parthenolide, andrographolide, ursolic acid and oleanolic acid.

30 Preferably, the diterpene lactone genus, as represented by Fig. 2 [B1] and specifically exemplified by andrographolide in Fig. 2 [B2] and the tritperpene genus, as represented by Fig. 2 [C1] and specifically exemplified by ursolic acid Fig. 2, [C2] or oleanolic acid, Fig. 2 [C3] as species is a pharmaceutical grade

preparation such as can be obtained commercially, for example, from Garden State Nutritionals, 8 Henderson Drive, West Caldwell NY 07006. Diterpene lactone, such as andrographolide can be obtained from *Andrographis paniculata*, while tripterpane such as ursolic acid or oleanolic acid are both found in a wide variety of botanicals. For example, ursolic acid can be sourced from *Adina piluifera*, *Agrimonia eupatoria*, *Arbutus unedo*, *Arctostaphylos uva-ursi*, *Artocarpus heterophyllus*, *Catalpa bignonioides*, *Catharanthus roseus*, *Chimaphila umbellata*, *Cornus florida*, *Cornus officinalis*, *Crataegus cuneata*, *Crataegus laevigata*, *Crataegus pinnatifida*, *Cryptostegia grandifolia*, *Elaeagnus pungens*, *Eriobotrya japonica*, *Eucalyptus citriodora*, *Forsythia suspensa*, *Gaultheria fragrantissima*, *Glechoma hederacea*, *Hedyotis diffusa*, *Helichrysum angustifolium*, *Humulus lupulus*, *Hyssopus officinalis*, *Ilex paraguariensis*, *Lavandula angustifolia*, *Lavandula latifolia*, *Leonurus cardiaca*, *Ligustrum japonicum*, *Limonia acidissima*, *Lycopus europeus*, *Malus domestica*, *Marubium vulgare*, *Melaleuca leucadendra*, *Melissa officinalis*, *Mentha spicata*, *Mentha x rotundifolia*, *Monarda didyma*, *Nerium oleander*, *Ocimum basilicum*, *Ocimum basilicum*, *Ocimum basilicum*, *Ocimum canum*, *Origanum majorana*, *Origanum vulgare*, *Plantago asiatica*, *Plantago major*, *Plectranthus amboinicus*, *Prunell vulgaris*, *Prunella vulgaris*, *Prunus cerasus*, *Prunus laurocerasus*, *Prunus persica*, *Prunus serotina* spp *serotina*, *Psidium guajava*, *Punica granatum*, *Pyrus communis*, *Rhododendron dauricum*, *Rhododendron ferrugineum*, *Rhododendron ponticum*, *Rosmarinus officinalis*, *Rubus fruticosus*, *Salvia officinalis*, *Salvia sclarea*, *Salvia triloba*, *Sambucus nigra*, *Sanguisorba officinalis*, *Satureja hortensis*, *Satureja montana*, *Sorbus aucubaria*, *Syringa vulgaris*, *Teucrium chamaedrys* *Teucrium polium*, *Teucrium* spp, *Thevetia peruviana*, *Thymus serpyllum*, *Thymus vulgaris*, *Uncaria tomentosa*, *Vaccinium corymbosum*, *Vaccinium myrtillus*, *Vaccinium vitis idaea*, *Verbena officinalis*, *Viburnum opulus* var. *opulus*, *Viburnum prunifolium*, *Vinca minor* or *Zizyphus jujuba*. Similarly, oleanolic acid is found in *Achyranthes aspera*, *Achyranthes bidentata*, *Adina piluifera*, *Ajpcynum cannabinum*, *Akebia quinata*, *Allium cepa*, *Allium sativum*, *Arctostaphylos uva-ursi*, *Calendula officinalis*,

Catharanthus roseus, Centaurium erythraea, Chenopodium album, Citrullus colocynthis, Cnicus benedictus, Cornus officinalis, Crataegus pinnatifida
5 *Cyperus rotundus, Daemonorops draco, Diospyros kaki, Elaeagnus pungens,*
Eleutherococcus senticosus, Eriobotrya japonica, Eugenia caryophyllata,
Forsythia suspensa, Glechoma hederacea, Harpagophytum procumbens, Hedera helix,, Hedyotis diffusa, Helianthus annuus, Hemsleya amabilis, Humulus lupulus, Hyssopus officinalis, Ilex rotunda, Lavandula latifolia, Leonurus cardiaca, Ligustrum japonicum, Ligustrum lucidum, Liquidambar orientalis,
10 *Liquidambar styraciflua, Loranthus parasiticus, Luffa aegyptiaca, Melaleuca leucadendra, Melissa officinalis, Mentha spicata, Mentha x rotundifolia,*
Momordica cochinchinensis, Myristica fragrans, Myroxylon balsamum, Nerium oleander, Ocimum suave, Ocimum basilicum, Olea europaea, Origanum majorana, Origanum vulgare, Paederia scandens, Panax ginseng, Panax japonicus, Panax quinquefolius, Patrinia scabiosaeefolia, Phytolacca americana,
15 *Plantago major, Plectranthus amboinicus, Prunella vulgaris, Prunus cerasus, Psidium guajava, Pulsatilla chinensis, Quisqualis indica, Rosmarinus officinalis, Salvia officinalis, Salvia sclarea, Salvia triloba, Sambucus nigra, Satureja hortensis, Satureja montana, Swertia chinensis, Swertia diluta, Swertia mileensis, Syzygium aromaticum, Thymus serpyllum, Thymus vulgaris, Trachycarpus fortunei, Uncaria tomentosa, Vaccinium corymbosum, Vaccinium myrtillus,*
20 *Viburnum prunifolium, Viscum album, Vitis vinifera, and Zizyphus jujuba.*

The preferred botanical sources for ursolic acid is a member selected from the group consisting of *Ligustrum japonicum, Plantago asiatica, Plantago major, Prunus species, Uncaria tomentosa, Zizyphus jujuba, Cornus officinalis, Eucalyptus citriodora, Forsythia suspensa, Lavandula latifolia, Malus domestica, Nerium oleander, Ocimum basilicum, Punica granatum, Pyrus communis, Rosmarinus officinalis, Salvia triloba, Sorbus aucubaria, Vaccinium myrtillus, Vaccinium vitis-idaea, and Viburnum opulus var. opulus.* The most preferred botanical sources for ursolic acid is a member selected from the group consisting of *Ligustrum japonicum, Plantago asiatica, Plantago major, Prunus species, Uncaria tomentosa, and Zizyphus jujuba.*

The preferred botanical sources for oleanolic acid is a member selected from the group consisting of *Eleutherococcus senticosus*, *Ligustrum japonicum*, *Ligustrum lucidum*, *Panax ginseng*, *Panax japonicus*, *Panax quinquefolius*, *Plantago major*, *Vitis vinifera*, *Zizyphus jujuba*, *Achyranthes bidentiata*, *Allium cepa*, *Allium sativum*, *Cornus officinalis*, *Daemonorops draco*, *Forsythia suspensa*, *Prunus cerasus*, *Quisqualis indica*, *Rosmarinus officinalis*, *Salvia triloba*, *Syzygium aromaticum*, *Thymus vulgaris*, *Uncaria tomentosa*, *Vaccinium corymbosum*, and *Vaccinium myrtillus*. The most preferred botanical sources for oleanolic acid is a member selected from the group consisting of
5 *Eleutherococcus senticosus*, *Ligustrum japonicum*, *Ligustrum lucidum*, *Panax ginseng*, *Panax japonicus*, *Panax quinquefolius*, *Plantago major*, *Vitis vinifera* and *Zizyphus jujuba*.

The pharmaceutical grade extract must pass extensive safety and efficacy procedures. Pharmaceutical grade andrographolide, ursolic acid or oleanolic acid refers to a preparation wherein the concentration of andrographolide, ursolic acid or oleanolic acid is greater than 90 percent by total weight of the preparation. As employed in the practice of the invention, the extract has an andrographolide, ursolic acid or oleanolic acid content of about 10 to 95 percent by weight. Preferably, the minimum andrographolide, ursolic acid or oleanolic acid content is greater than 50 percent by weight. The pharmaceutical grade extracts are particularly preferred. Without limiting the invention, it is anticipated that andrographolide, ursolic acid or oleanolic acid act to inhibit the generation of reactive oxygen intermediates (ROI) from AA metabolism and thereby prevent an increase in the rate of transcription of the COX-2 gene by the
15 transcriptional regulatory factor NF-kappa B.
20 25

Without limiting the invention, the action of the diterpene lactones or triterpenes is thought to inhibit COX-2 enzyme activity by providing a dual, synergistic effect with diterpene triepoxide lactones or sesquiterpene lactones. It has also been discovered that the combination of the primary components,
30 diterpene triepoxide lactones and sesquiterpene lactones or a combination of species within either genus also provides for a synergistic effect on the activity

of each other. By inhibiting both the generation of free radicals from the production of prostaglandins as well as COX-2 enzyme activity, the second compound selected from the group consisting of diterpene lactones, triterpenes, diterpene triepoxid lactones and sesquiterpene lactones increases the anti-inflammation activity of diterpene triepoxide lactones or sesquiterpene lactones. 5 The result of the combinations of this invention is a more selective effect on the activity of COX-2 at lower doses of diterpene triepoxide lactones or sesquiterpene lactones than would normally be required. By decreasing the dose of diterpene triepoxid lactones or sesquiterpene lactones to achieve the desired 10 COX-2 inhibition, the probability of side effects from this compound decreases almost exponentially. The second compound can also provide hepatoprotection, antitumor promotion, antihyperlipidemia, antihyperglycemia, and protection against ulcer formation from COX-1 inhibiting agents.

A daily dose (mg/kg-day) of the present dietary supplement would be 15 formulated to deliver, per kg body weight of the animal, about 0.001 to 3.0 mg diterpene triepoxid lactones or/and about 0.05 to 5 mg sesquiterpene lactones, and about 0.5 to 20.0 mg diterpene lactones or triterpenes.

The composition of the present invention for topical application would contain one of the following: about 0.001 to 1 wt%, preferably 0.01 to 1 wt% 20 diterpene triepoxid lactones or sesquiterpene lactones, and about 0.025 to 1 wt%, preferably 0.05 to 1 wt% diterpene lactones or triterpenes.

The preferred composition of the present invention would produce serum concentrations in the following range: 0.01 to 10 nM diterpene triepoxid lactones, and 0.001 to 10 μ M sesquiterpene lactone, diterpene lactones or 25 triterpenes.

In addition to the combination of the active ingredients selected from the group consisting of diterpene triepoxid lactones, sesquiterpene lactone, diterpene lactones, triterpenes or derivatives thereof, the present composition for dietary application may include various additives such as other natural 30 components of intermediary metabolism, vitamins and minerals, as well as inert

ingredients such as talc and magnesium stearate that are standard excipients in the manufacture of tablets and capsules.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, sweeteners and the like. These pharmaceutically acceptable carriers may be prepared from a wide range of materials including, but not limited to, diluents, binders and adhesives, lubricants, disintegrants, coloring agents, bulking agents, flavoring agents, sweetening agents and miscellaneous materials such as buffers and absorbents that may be needed in order to prepare a particular therapeutic composition. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in the present composition is contemplated. In one embodiment, talc and magnesium stearate are included in the present formulation. When these components are added they are preferably, the Astac Brand 400 USP talc powder and the veritable grade of magnesium stearate. Other ingredients known to affect the manufacture of this composition as a dietary bar or functional food can include flavorings, sugars, amino-sugars, proteins and/or modified starches, as well as fats and oils.

The dietary supplements, lotions or therapeutic compositions of the present invention can be formulated in any manner known by one of skill in the art. In one embodiment, the composition is formulated into a capsule or tablet using techniques available to one of skill in the art. In capsule or tablet form, the recommended daily dose for an adult human or animal would preferably be contained in one to six capsules or tablets. However, the present compositions may also be formulated in other convenient forms, such as an injectable solution or suspension, a spray solution or suspension, a lotion, gum, lozenge, food or snack item. Food, snack, gum or lozenge items can include any ingestible ingredient, including sweeteners, flavorings, oils, starches, proteins, fruits or fruit extracts, vegetables or vegetable extracts, grains, animal fats or proteins. Thus, the present compositions can be formulated into cereals, snack items such as chips, bars, gum drops, chewable candies or slowly dissolving lozenges.

The present invention contemplates treatment of all types of inflammation-based diseases, both acute and chronic. The present formulation reduces the inflammatory response and thereby promotes healing of, or prevents further damage to, the affected tissue. A pharmaceutically acceptable carrier may also be used in the present compositions and formulations.

5 Table 2 below provides a list of diseases in which COX-2 enzyme expression and activity may play a significant role and therefore are appropriate targets for normalization or treatment by the invention.

TABLE 2

Disease	Tissue Affected
Addison's Disease	Adrenal
Allergies	Inflammatory cells
Alzheimer Disease	Nerve cells
Arthritis	Inflammatory cells
Atherosclerosis	Vessel wall
Colon Cancer	Intestine
Crohn's Disease	Intestine
Diabetes (type I)/type II	Pancreas
Eczema	Skin/Inflammatory cells
Graves' Disease	Thyroid
Guillain-Barre Syndrome	Nerve cells
Inflammatory Bowel Disease	Intestine
Leukemia	Immune cells
Lymphomas	Immune cells
Multiple Sclerosis	Nerve cells
Myasthenia Gravis	Neuromuscular junction
Osteoarthritis	Joint lining
Psoriasis	Skin
Primary Biliary Cirrhosis	Liver
Rheumatoid Arthritis	Joint lining
Solid Tumors	Various
Systemic Lupus Erythematosis	Multiple tissues
Uveitis	Eye

According to the present invention, the animal may be a member selected from the group consisting of humans, non-human primates, such as dogs, cats, birds, horses, ruminants or other warm blooded animals. The invention is directed primarily to the treatment of human beings. Administration can be by any method available to the skilled artisan, for example, by oral, topical, transdermal, transmucosal, or parenteral routes.

The following examples are intended to illustrate but not in any way limit the invention:

EXAMPLE 1

Inhibition of Cyclooxygenase-2 Enzyme Expression in Human T Cells by
5 Triptolide and Parthenolide

This example hypothetically illustrates the effect of triptolide and parthenolide on inducible cyclooxygenase COX-2 in cultured Jurkat cells. It is found that triptolide alone may decrease the expression of COX-2 protein in PMA stimulated cells and that parthenolide has little effect in the dose-range 10 tested. In the presence of arachidonic acid (AA), the effectiveness of triptolide is markedly reduced. However, a combination of the two compounds exerts a powerful inhibition of the expression of COX-2 in the presence and absence of AA, with no observable signs of toxicity.

15 **Chemicals:** Anti-COX-2 antibodies may be purchased from Upstate Biotechnology (Lake Placid, NY). Triptolide and parthenolide may be obtained from Sigma (St. Louis, MO). Arachidonic acid (AA), PMA and all other chemical may also be purchased from Sigma and are of the highest purity commercially available.

20 **Human T cell lines:** The Jurkat cell line is useful as a model for human T cells and may be obtained from the American Type Culture Collection (Bethesda, MD). COX-2 is inducible in the Jurkat cell by PMA.

25 **Cell plating:** The Jurkat cells are propagated in suspension according to the instructions of the supplier. For experimentation, cells are seeded from a log-phase culture at a density of 1×10^5 cells per mL in 100 mm plates, 20 mL per plate, 3 plates per treatment. Serum concentration in the test medium is maintained at 0.5%. After 24 hours, the phytohemagglutinin (PHA) or PHA/AA combinations are added to the cell cultures, in 10 μ L aliquots, to achieve effective concentrations.

30 **Gel Electrophoresis:** Sodium dodecyl sulfate polyacryamide gel electrophoresis (PAGE) is performed using 10% polyacrylamide gels as described by Laemmli, U.K. and Favre, M. (J. Mol. Biol. (1973) 80:575) with

the modification that the cell lysates (100 µg/lane) are heated at 100°C for three minutes.

5 **Immunoblotting:** The immunoblotting is performed as described by Tobin et al. (Proc. Nat. Acad. Sci. USA (1979) 76:4350), however, Milliblot SDE electroblot apparatus (Millipore, Bedford, MA) is used to transfer proteins from the polyacrylamide gels to an Immobilon® membrane filter. Complete transfers are accomplished in 25-30 minutes at 500 mA. Membranes used for blotting are blocked by incubating in TBS (Tris buffered saline, 50 mM tris, 150 mM NaCl, pH 7.5) containing 5% nonfat dry milk for 30 minutes at room temperature. COX-2 protein is visualized by incubation of the blots with the anti-COX-2 antibody in TBST (0.5% Tween 20 in TBS) for two hours and then a second incubation at room temperature with alkaline phosphatase-conjugated secondary antibody diluted 1:1000 in TBST for two hours. The enzymatic reaction is developed for 15 minutes. The molecular weight of COX-2 is estimated by adding a molecular weight standard to reference lanes and staining the membrane filters with amido black 10B.

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Blots are translated into TIFF-formatted files with a Microtech 600GS scanner and quantified using Scan Analysis (BIOSOFT, Cambridge UK). Summary scans are then printed and peak heights are measured directly from the figure. One density unit (Du) is defined as one mm of the resulting peak height.

20

25 **Protein determination:** Spectrophotometric determination of protein concentration is determined with bicinchoninic acid as reported by Smith et al. (Anal. Biochem. (1985) 150:76).

FIG. 3 provides a schematic for the experimental design in which Jurkat cells are stimulated with PHA in the absence and present of arachidonic acid. Triptolide or parthenolide or a compound selected from the group consisting of andrographolide, ursolic acid and oleanolic acid alone, or a combination of triptolide or parthenolide and a compound selected from the group consisting of andrographolide, ursolic acid and oleanolic acid are added in a volume of 10 µL immediately to the medium immediately following the PHA treatment.

30 Appropriate controls receive solvent only. Final concentrations of triptolide or

parthenolide are 0, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, 100, 500 and 1,000 nM. For the mixtures, the first seven doses are simply combined. For example, the first does of the combined treatment contains 0.01 nM triptolide and 0.01 nM oleanolic acid. Twenty-four hours after treatment, the cells are harvested, lysed and western blotting is done for the determination of COX-2 protein expression.

FIG. 4(a) is a line graph depicting the percent inhibition of COX-2 enzyme protein expression by triptolide, parthenolide and the combination of triptolide with parthenolide in the absence and presence of arachidonic acid. It is observed that triptolide functions to inhibit the expression of inducible cyclooxygenase 2 enzyme in the Jurkat cell line in the absence of arachidonic acid; and that this activity is enhanced more than 10-fold by parthenolide. Parthenolide alone does not inhibit COX-2 expression at physiologically relevant doses. In the presence of parthenolide, the inhibition of inducible COX-2 by triptolide is nearly complete, even at very low concentrations. In the presence of arachidonic acid, triptolide inhibition of COX-2 enzyme protein is compromised, but restored in the presence of parthenolide.

EXAMPLE 2

Inhibition of Cyclooxygenase-2 Enzyme Expression in Human T Cells by
20 Triptolide and Andrographolide

This example hypothetically illustrates the effect of triptolide and andrographolide on inducible cyclooxygenase COX-2 in cultured Jurkat cells.

The experiment is performed as described in EXAMPLE 1, except that the second compound is andrographolide, which may be obtained from Sigma (St. Louis, MO).

FIG. 4(b) is a line graph depicting the percent inhibition of COX-2 enzyme protein expression by triptolide, andrographolide and the combination of triptolide with andrographolide in the absence and presence of arachidonic acid. It is observed that triptolide functions to inhibit the expression of inducible cyclooxygenase 2 enzyme in the Jurkat cell line in the absence of arachidonic acid, and that this activity is enhanced more than 10-fold by

andrographolide alone does not inhibit COX-2 expression at physiologically relevant doses. In the presence of andrographolide the inhibition of inducible COX-2 by triptolide is nearly complete, even at very low concentrations. In the presence of arachidonic acid, triptolide inhibition of COX-2 enzyme protein is compromised, but restored in the presence of andrographolide.

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EXAMPLE 3

Inhibition of Cyclooxygenase-2 Enzyme Expression in Human T Cells by Triptolide and Oleanolic Acid

This example hypothetically illustrates the effect of triptolide and oleanolic acid on the inducible cyclooxygenase COX-2 in cultured Jurkat cells. The experiment is performed as described in EXAMPLE 1, except that the second compound is oleanolic acid, which may be obtained from Sigma (St. Louis, MO).

FIG. 4(c) is a line graph depicting the percent inhibition of COX-2 enzyme protein expression by triptolide, oleanolic acid and the combination of triptolide with oleanolic acid in the absence and presence of arachidonic acid. It is clearly demonstrated that triptolide functions to inhibit the expression of inducible cyclooxygenase 2 enzyme in the Jurkat cell line in the absence of arachidonic acid, and that this activity is enhanced more than 10-fold by oleanolic acid. Oleanolic acid alone does not inhibit COX-2 expression at physiologically relevant doses. In the presence of oleanolic acid the inhibition of inducible COX-2 by triptolide is nearly complete, even at very low concentrations. In the presence of arachidonic acid, triptolide inhibition of COX-2 enzyme protein is compromised, but restored in the presence of oleanolic acid.

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EXAMPLE 4

Inhibition of Cyclooxygenase-2 Enzyme Expression in Human T Cells by Triptolide and Ursolic acid

This example hypothetically illustrates the effect of triptolide and ursolic acid on the inducible cyclooxygenase COX-2 in cultured Jurkat cells. The

30

experiment is performed as described in EXAMPLE 1, except that the second compound is ursolic acid, which may be obtained from Sigma (St. Louis, MO).

FIG. 4(d) is a line graph depicting the percent inhibition of COX-2 enzyme protein expression by triptolide, ursolic acid and the combination of triptolide with ursolic acid in the absence and presence of arachidonic acid. It is observed that triptolide functions to inhibit the expression of inducible cyclooxygenase 2 enzyme in the Jurkat cell line in the absence of arachidonic acid, and that this activity is enhanced more than 10-fold by ursolic acid.

Ursolic acid alone does not inhibit COX-2 expression at physiologically relevant doses. In the presence of ursolic acid the inhibition of inducible COX-2 by triptolide is nearly complete, even at very low concentrations. In the presence of arachidonic acid, triptolide inhibition of COX-2 enzyme protein is compromised, but restored in the presence of ursolic acid.

15

EXAMPLE 5

Inhibition of Cyclooxygenase-2 Enzyme Expression in Human T Cells by Parthenolide and Andrographolide

This example hypothetically illustrates the effect of parthenolide and andrographolide on the inducible cyclooxygenase COX-2 in cultured Jurkat cells.

The experiment is performed as described in EXAMPLE 1, except that the first compound is parthenolide and the second compound is andrographolide. It is found that both parthenolide and andrographolide have little effect on decreasing the expression of COX-2 protein in PMA stimulated Jurkat cells in the dose-range tested. However, combinations of the two compounds exerted a powerful inhibition of the expression of COX-2 in the presence and absence of AA with no observable signs of toxicity.

FIG. 4(e) is a line graph depicting the percent inhibition of COX-2 enzyme protein expression by parthenolide, andrographolide and the combination of parthenolide with andrographolide (Combination) in the absence and presence of arachidonic acid. It is observed that, within the dose-range

tested, parthenolide does not effectively function to inhibit the expression of inducible cyclooxygenase 2 enzyme in the Jurkat cell line in the absence or presence of arachidonic acid. Furthermore, andrographolide alone does not inhibit COX-2 expression at physiologically relevant doses. In the presence of andrographolide, the inhibition of inducible COX-2 by parthenolide is nearly complete, even at very low concentrations both with and without arachidonic acid.

EXAMPLE 6

10 Inhibition of Cyclooxygenase-2 Enzyme Expression in Human T Cells by
Parthenolide and Oleanolic Acid

This example hypothetically illustrates the effect of parthenolide and oleanolic acid on the inducible cyclooxygenase COX-2 in cultured Jurkat cells. The experiment is performed as described in EXAMPLE 5, except that the second compound is oleanolic acid.

FIG.4(f) is a line graph depicting the percent inhibition of COX-2 enzyme protein expression by parthenolide, oleanolic acid and the combination of parthenolide with oleanolic acid (Combination) in the absence and presence of arachidonic acid. It is observed that, within the dose-range tested, parthenolide does not effectively function to inhibit the expression of inducible cyclooxygenase 2 enzyme in the Jurkat cell line in the absence or presence of arachidonic acid. Furthermore, oleanolic acid alone does not inhibit COX-2 expression at physiologically relevant doses. In the presence of oleanolic acid inhibition of inducible COX-2 by parthenolide is nearly complete, even at very low concentrations of each test material both with and without arachidonic acid.

EXAMPLE 7**Inhibition of Cyclooxygenase-2 Enzyme Expression in Human T Cells by
Parthenolide and Ursolic acid**

This example hypothetically illustrates the effect of parthenolide and 5 ursolic acid on the inducible cyclooxygenase COX-2 in cultured Jurkat cells.

The experiment is performed as described in EXAMPLE 5, except that the second compound is ursolic acid.

FIG.4(g) is a line graph depicting the percent inhibition of COX-2 10 enzyme protein expression by parthenolide, ursolic acid and the combination of parthenolide with ursolic acid (Combination) in the absence and presence of arachidonic acid. It is observed that, within the dose-range tested, parthenolide does not effectively function to inhibit the expression of inducible 15 cyclooxygenase 2 enzyme in the Jurkat cell line, in the absence or presence of arachidonic acid. Furthermore, ursolic acid alone does not inhibit COX-2 expression at physiologically relevant doses. In the presence of ursolic acid 20 inhibition of inducible COX-2 by parthenolide is nearly complete, even at very low concentrations both with and without arachidonic acid.

As represented in the above EXAMPLES 1-7, the specific and nearly 25 complete inhibition of COX-2 enzyme expression by combinations of triptolide or parthenolide with a second compound selected from the group consisting of triptolide, parthenolide, andrographolide, ursolic acid and oleanolic acid, with non-toxicity to other cellular functions, is a surprising and unexpected aspect of the present invention. The compositions of the present invention may exert beneficial effects in processes in which de novo COX-2 expression is involved and, in a broader sense, in pathological situations in which genes under nuclear factor-kappaB control are up-regulated.

EXAMPLE 8**Normalization of Joint Functioning Following Trauma**

30 A representative composition of the present invention as a dietary supplement would be in an oral formulation, i.e. tablets, that would supply one

of the following combinations: (a) 0.01 mg triptolide/kg per day and 5.0 mg parthenolide/kg per day; (b) 0.01 mg triptolide/kg per day and 6.0 mg andrographolide/kg per day; (c) 1 mg parthenolide/kg per day and 6.0 mg ursolic acid/kg per day; (d) 0.01 mg triptolide/kg per day and 6.0 mg ursolic acid/kg per day; (e) 0.01 mg triptolide/kg per day and 6.0 mg oleanolic acid/kg per day; (f) 1 mg parthenolide/kg per day and 6.0 mg oleanolic acid/kg per day; or (g) 1 mg parthenolide/kg per day and 6.0 mg andrographolide/kg per day. Normalization of joint movement following physical trauma due to exercise or repetitive movement stress would be expected to occur following two to ten doses. This result would be expected in all warm-blooded animals.

EXAMPLE 9

Clinical Effectiveness of Lotion Formulations in the Treatment of Acne Rosacea

A lotion designed to contain one of the following: (a) 0.1% wt triptolide and 0.1% wt parthenolide; (b) 0.1% wt triptolide and 0.5% andrographolide; (c) 0.1% wt triptolide and 0.5% ursolic acid; (d) 0.1% wt triptolide and 0.5% oleanolic acid; (e) 0.1% wt parthenolide and 0.5% andrographolide; (f) 0.1% wt parthenolide and 0.5% ursolic acid or (g) 0.1% wt parthenolide and 0.5% oleanolic acid, is applied to affected areas of patients who have exhibited acne rosacea as diagnosed by their own practitioner and confirmed by an independent board-certified dermatologist. Self-evaluation tests are administered one week prior to the study to quantify the surface area affected and redness. In addition, similar variables are scored by the professional clinical staff not aware of the patients treatment status. These evaluations are repeated on Days 0, 7, 14 and 21.

Patients are randomly assigned to the test formulation or a placebo at the start of the study. The test formulation and placebo are applied to the affected area one or two times per day. Treatment for health conditions such as diabetes, hypertension, etc. is allowed during the study. Scores are statistically compared between the test formulation and the placebo for each of the four observational periods. Patients treated with the combination composition of the present

invention in a lotion formulation are considered improved if the patients' scores improve by greater than 20% from the pre-test scores within each category evaluated. The percentage of persons exhibiting improvement are compared between the combination formulations and the placebo control. The difference between the two groups is considered statistically significant if the probability of rejecting the null hypothesis when true is less than five percent.

EXAMPLE 10

Clinical Effectiveness of Lotion Formulations in the Treatment of Psoriasis

This example is performed in the same manner as described in Example 9, except that the composition is applied to affected areas of patients who have exhibited psoriasis as diagnosed by their own practitioner and confirmed by an independent board-certified dermatologist. Self-evaluation tests are administered one week prior to the study to quantify the surface area affected and skin condition. In addition, similar variables are scored by the professional clinical staff not aware of the patients treatment status. These evaluations are repeated on Days 0, 7, 30 and 60.

Patients are randomly assigned to the test formulation or placebo at the start of the study. The test formulation and placebo are applied to the affected area one or two times per day. Treatment for health conditions such as diabetes, hypertension, etc. is allowed during the study. Scores are statistically compared between the test formulation and the placebo for each of the four observational periods. Patients treated with the triptolide/oleanolic acid lotion formulation are considered improved if the patients' scores improve by greater than 20% from the pre-test scores within each category evaluated. The percentage of persons exhibiting improvement is compared between the triptolide/oleanolic acid formulation and the placebo control. The difference between the two groups is considered statistically significant if the probability of rejecting the null hypothesis when true is less than five percent.

EXAMPLE 11**Clinical Effectiveness of an Oral Triptolide/Oleanolic Acid Formulation in
the Treatment of Alzheimer's Disease**

An oral triptolide/oleanolic acid formulation as described in Example 8
5 is administered to patients who have manifested an early stage of Alzheimer's
Disease (AD), as diagnosed by their own practitioner and confirmed by an
independent board-certified neurologist. Two weeks before the clinical trial, the
patients undergo appropriate psychoneurological tests such as the Mini Mental
Status Exam (MMSE), the Alzheimer Disease Assessment Scale (ADAS), the
10 Boston Naming Test (BNT), and the Token Test (TT). Neuropsychological tests
are repeated on Day 0, 6 weeks and 3 months of the clinical trials. The tests are
performed by neuropsychologists who are not aware of the patient's treatment
regimen.

Patients are randomly assigned to the test formulations or a placebo at
15 the start of the study. The test formulation and placebo are taken orally one or
two times per day. Treatment for conditions such as diabetes, hypertension, etc.
is allowed during the study. Scores are statistically compared between the test
formulation and the placebo for each of the three observational periods.
Without treatment the natural course of AD is significant deterioration in the test
20 scores during the course of the clinical trial. Patients treated with the
triptolide/oleanolic acid formulation are considered improved if the patients'
scores remain the same or improve during the course of the clinical trial.

EXAMPLE 12**Clinical Effectiveness of an Oral Triptolide/Oleanolic Acid Formulation
in the Treatment and Prevention of Colon Cancer**

An oral triptolide/oleanolic acid formulation as described in Example 8
is administered to patients who have manifested an early stage of colon cancer
as diagnosed by their own practitioner and confirmed by an independent board-
30 certified oncologist.

Patents are randomly assigned to the test formulation or placebo at the start of the study. The test formulation and placebo are taken orally one or two times per day. Treatment for conditions such as diabetes, hypertension, etc. is allowed during the study. Endoscopic evaluations are made at one, two, six and 5 twelve months. Evidence of reappearance of the tumor during any one of the four follow-up clinical visits is considered a treatment failure. The percentage of treatment failures is compared between the triptolide/oleanolic acid formulation and the placebo control. The difference between the two groups is considered statistically significant if the probability of rejecting the null 10 hypothesis when true is less than five percent.

EXAMPLE 13

Clinical Effectiveness of an Oral Triptolide/Oleanolic Acid Formulation in the Treatment of Irritable Bowel Syndrome

A preferred oral triptolide/oleanolic acid formulations as described in 15 Example 8 is administered to patients who have manifested irritable bowel syndrome as diagnosed by their own practitioner. Normal bowel functioning is restored within 24 hours.

EXAMPLE 14

Normalization of Joint Functioning in Osteoarthritis

Using compositions described in Example 8, normalization of joint stiffness due to osteoarthritis occurs following five to twenty doses, in the presence or absence of glucosamine or chondroitin sulfate. In addition, the composition 25 does not interfere with the normal joint rebuilding effects of these two proteoglycan constituents, unlike traditional non-steroidal anti-inflammatory agents.

EXAMPLE 15**Inhibition of COX-2 Enzyme Production of Prostaglandin E2 in Murine
B Cells by Parthenolide and Andrographolide**

This example illustrates the superior COX-2 inhibitory potency and
5 selectivity of the combination of parthenolide and andrographolide of the
present invention compared to parthenolide or andrographolide alone.

Inhibition of COX-2 Mediated Production of PGE2 in RAW 264.7 Cells

Equipment - balancer, analytical, Ohaus Explorer (Ohaus Model
10 #EO1140, Switzerland), biosafety cabinet (Forma Model #F1214, Marietta,
Ohio), pipettor, 100 to 1000 µL (VWR Catalog #4000-208, Rochester, NY), cell
hand tally counter (VWR Catalog #23609-102, Rochester, NY), CO₂ incubator
(Forma Model #F3210, Marietta, Ohio), hemacytometer (Hausser Model #1492,
Horsham, PA), microscope, inverted (Leica Model #DM IL, Wetzlar, Germany),
15 multichannel pipettor, 12-Channel (VWR Catalog #53501-662, Rochester, NY),
Pipet Aid (VWR Catalog #53498-103, Rochester, NY), Pipettor, 0.5 to 10 µL
(VWR Catalog #4000-200, Rochester, NY), pipettor, 100 to 1000 µL (VWR
Catalog #4000-208, Rochester, NY), pipettor, 2 to 20 µL (VWR Catalog #4000-
202, Rochester, NY), pipettor, 20 to 200 µL (VWR Catalog #4000-204,
20 Rochester, NY), PURELAB Plus Water Polishing System (U.S. Filter, Lowell,
MA), refrigerator, 4°C (Forma Model #F3775, Marietta, Ohio), vortex mixer
(VWR Catalog #33994-306, Rochester, NY), water bath (Shel Lab Model
#1203, Cornelius, OR).

Cells, Chemicals, Reagents and Buffers - Cell scrapers (Corning Catalog
25 #3008, Corning, NY), dimethylsulfoxide (DMSO) (VWR Catalog #5507,
Rochester, NY), Dulbecco's Modification of Eagle's Medium (DMEM)
(Mediatech Catalog #10-013-CV, Herndon, VA), fetal bovine serum, heat
inactivated (FBS-HI) (Mediatech Catalog #35-011-CV, Herndon, VA),
lipopolysaccharide (LPS)(Sigma Catalog #L-2654, St. Louis, MO), microfuge
30 tubes, 1.7 mL (VWR Catalog #20172-698, Rochester, NY),
penicillin/streptomycin (Mediatech Catalog #30-001-CI, Herndon, VA), pipet

tips for 0.5 to 10 μ L pipettor (VWR Catalog #53509-138, Rochester, NY), pipet tips for 100-1000 μ L pipettor (VWR Catalog #53512-294, Rochester, NY), pipet tips for 2-20 μ L and 20-200 μ L pipettors (VWR Catalog #53512-260, Rochester, NY), pipets, 10 mL (Becton Dickinson Catalog #7551, Marietta, OH), pipets, 2 mL (Becton Dickinson Catalog #7507, Marietta, OH, pipets, 5 mL (Becton Dickinson Catalog #7543, Marietta, OH), RAW 264.7 Cells (American Type Culture Collection Catalog #TIB-71, Manassas, VA), test compounds (liquid CO₂ hops extract from Hopunion, Yakima, WA), tissue culture plates, 96-well (Becton Dickinson Catalog #3075, Franklin Lanes, NJ),
10 Ultra-pure water (Resistance =18 megaOhm-cm deionized water).

15 *General Procedure* - RAW 264.7 cells, obtained from ATCC, were grown in DMEM medium and maintained in log phase growth. The DMEM growth medium was made as follows: 50 mL of heat inactivated FBS and 5 mL of penicillin/streptomycin were added to a 500 mL bottle of DMEM and stored at 4°C. This was warmed to 37°C in a water bath before use and for best results should be used within three months.

20 On day one of the experiment, the log phase 264.7 cells were plated at 8 $\times 10^4$ cells per well in 0.2 mL growth medium per well in a 96-well tissue culture plate. After 6 to 8 hours post plating, 100 μ L of growth medium from each well was removed and replaced with 100 μ L fresh medium. A 1.0 mg/mL solution of LPS, which was used to induce the expression of COX-2 in the RAW 264.7 cells, was prepared by dissolving 1.0 mg of LPS in 1 mL DMSO. It was mixed until dissolved and stored at 4°C. Immediately before use, it was thawed at room temperature or in a 37 °C water bath.

25 On day two of the experiment, the test materials were prepared as 1000X stock in DMSO. For example, if the final concentration of the test material was to be 10 μ g/mL, a 10 mg/mL stock was prepared by dissolving 10 mg of the test material in 1 mL of DMSO. Fresh test materials were prepared on day 2 of the experiment. In 1.7 mL microfuge tubes, 1 mL DMEM without FBS was added to obtain test concentrations of 0.05, 0.10, 0.5, and 1.0 μ g/mL. 2 μ L of the 1000X DMSO stock of the test material was added to the 1 mL of medium
30

without FBS. The tube contained the final concentration of the test material was concentrated 2-fold. The tube was placed in incubator for 10 minutes to equilibrate.

One-hundred mL of medium was removed from each well of the cell plates prepared on day one. One-hundred mL of equilibrated 2X final concentration the test compounds were added to cells and incubated for 90 minutes. LPS in DMEM without FBS was prepared by adding 44 µL of the 1 mg/mL DMSO stock to 10 mL of medium. For each well of cells to be stimulated, 20 µL of LPS (final concentration of LPS is 0.4 µg/mL of LPS) was added. The LPS stimulation was continued for 24 hours, after which the supernatant medium from each well was transferred to a clean microfuge tube for determination of the PGE2 content in the medium.

Determination of COX-1 Enzyme Inhibition by parthenolide and
andrographolide

The ability of a test material to inhibit COX-1 synthesis of PGE2 was determined essentially as described by Noreen, Y., et al. (J. Nat. Prod. 61, 2-7, 1998).

Equipment - balancer (2400 g, Acculab VI-2400, VWR Catalog #11237-300, Rochester, NY), balancer, analytical, Ohaus Explorer (Ohaus Model #EO1140, Switzerland), biosafety cabinet (Forma Model #F1214, Marietta, Ohio), Freezer, -30°C (Forma Model #F3797), Freezer, -80°C Ultralow (Forma Model #F8516, Marietta, OH), heated stirring plate (VWR Catalog #33918-262, Rochester, NY), ice maker (Scotsman Model #AFE400A-1A, Fairfax, SC), multichannel pipettor, 12-Channel (VWR Catalog #53501-662, Rochester, NY), Multichannel Pipettor, 8-Channel (VWR Catalog #53501-660, Rochester, NY), orbital shaker platform (Scienceware #F37041-0000, Pequannock, NJ), pH meter (VWR Catalog #33221-010, Rochester, NY), pipet aid (VWR Catalog #53498-103, Rochester, NY), pipettor, 0.5 to 10 µL (VWR Catalog #4000-200, Rochester, NY), pipettor, 100 to 1000 µL (VWR Catalog #4000-208, Rochester, NY), pipettor, 2 to 20 µL (VWR Catalog #4000-202, Rochester, NY), pipettor,

20 to 200 µL (VWR Catalog #4000-204, Rochester, NY), PURELAB Plus Water Polishing System (U.S. Filter, Lowell, MA), refrigerator, 4°C (Forma Model #F3775, Marietta, Ohio), vacuum chamber (Sigma Catalog #Z35, 407-4, St. Louis, MO), vortex mixer (VWR Catalog #33994-306, Rochester, NY)

5 *Supplies and Reagents* - 96-Well, round-bottom plate (Nalge Nunc #267245, Rochester, NY), arachidonic acid (Sigma Catalog #A-3925, St. Louis, MO), centrifuge tubes, 15 mL, conical, sterile (VWR Catalog #20171-008, Rochester, NY), COX-1 enzyme (ovine) 40,000 units/mg (Cayman Chemical Catalog #60100, Ann Arbor, MI), dimethylsulfoxide (DMSO) (VWR Catalog #5507, Rochester, NY), ethanol 100% (VWR Catalog #MK701908, Rochester, NY), epinephrine (Sigma Catalog #E-4250, St. Louis, MO), glutathione (reduced) (Sigma Catalog # G-6529, St. Louis, MO), graduated cylinder, 1000 mL (VWR Catalog #24711-364, Rochester, NY), hematin (porcine) (Sigma catalog # H-3281, St. Louis, MO), hydrochloric acid (HCl) (VWR Catalog #VW3110-3, Rochester, NY), KimWipes (Kimberly Clark Catalog #34256, Roswell, GA), microfuge tubes, 1.7 mL (VWR Catalog #20172-698, Rochester, NY), NaOH (Sigma Catalog #S-5881, St. Louis, MO), pipet tips for 0.5 to 10 µL pipettor (VWR Catalog #53509-138, Rochester, NY), pipet tips for 100-1000 µL pipettor (VWR Catalog #53512-294, Rochester, NY), pipet tips for 2-20 µL and 20-200 µL pipettors (VWR Catalog #53512-260, Rochester, NY), prostaglandin E2 (Sigma Catalog # P-5640, St. Louis, MO), prostaglandin F2alpha (Sigma Catalog # P-0424, St. Louis, MO), stir bar, magnetic (VWR Catalog #58948-193, Rochester, NY), storage bottle, 1000 mL (Corning Catalog #1395-1L, Corning, NY), storage bottle, 100 mL (Corning Catalog #1395-100, Corning, NY), CO₂ extract of hops (Hopunion, Yakima, WA), Tris-HCl (Sigma Catalog #T-5941, St. Louis, MO), ultra-pure water (Resistance =18 megaOhm-cm deionized water).

20 *General Procedure* - Oxygen-free 1.0M Tris-HCl buffer (pH 8.0) was prepared as follows. In a 1000 mL beaker, 12.11g Trizma HCl was dissolved into 900 mL ultra-pure water. The beaker was placed on a stir plate with a stir

bar. NaOH was added until the pH reached 8.0. The volume was adjusted to a final volume of 1000mL and stored in a 1000 mL storage bottle.

5 The Tris-HCl buffer was placed into a vacuum chamber with the top loosened and the air pump was turned on until the buffer stopped bubbling. The vacuum chamber was then turned off and the storage bottle was tightly covered. This step was repeated each time when oxygen-free Tris-HCl buffer was used.

10 One mL cofactor solution was prepared by adding 1.3 mg (-) epinephrine, 0.3 mg reduced glutathione and 1.3 mg hematin to 1 mL oxygen free Tris-HCl buffer. The solutions of the test material were prepared as needed. i.e. 10 mg of aspirin was weighed and dissolved into 1 mL DMSO.

15 Enzymes, i.e. prostaglandin E2 or prostaglandin F2alpha, were dissolved in oxygen free Tris-HCl buffer as follows, i.e. on ice, 6.5 μ L of enzyme at 40,000 units/mL was taken and added to 643.5 μ L of oxygen free Tris-HCl buffer. This enzyme solution is enough for 60 reactions. The COX-1 enzyme solution was prepared as follows: In a 15 mL centrifuge tube, 10 μ L COX-1 enzyme at 40,000 units/mL was added to oxygen free Tris-HCl with 50 μ L of the cofactor solution per reaction. The mixture was incubated on ice for 5 minutes. For 60 reactions, 650 μ L enzyme were added in oxygen free Tris-HCl buffer with 3.25 mL cofactor solution.

20 Sixty microliters of the enzyme solution were combined with 20 μ L of the test solution in each well of a 96 well plate. Final concentrations of the test solutions were 100, 50, 25, 12.5, 6.25 and 3.12 μ g/mL. The plates were preincubated on ice for 10 minutes. Twenty μ L arachidonic acid (30 μ M) was added and incubated for 15 minutes at 37°C.

25 Two M HCl was prepared by diluting 12.1 N HCl. in a 100 mL storage bottle. 83.5 mL ultra-pure water was added and then 16.5 mL 12.1 N HCl was added. It was stored in a 100 mL storage bottle and placed in the Biosafety cabinet. The reaction was terminated by adding 10 μ L 2 M HCl. The final solution was used as the supernatant for the PGE₂ assay.

The procedure followed was that essentially described by Hamberg, M. and Samuelsson, B. (*J. Biol. Chem.* 1971, 246, 6713-6721); however a commercial, nonradioactive procedure was employed.

5 *Equipment* - freezer, -30°C (Forma Model #F3797), heated stirring plate (VWR Catalog #33918-262, Rochester, NY), multichannel pipettor, 12-Channel (VWR Catalog #53501-662, Rochester, NY), orbital shaker platform (Scienceware #F37041-0000, Pequannock, NJ), Pipet Aid (VWR Catalog #53498-103, Rochester, NY), pipettor, 0.5 to 10 µL (VWR Catalog #4000-200, Rochester, NY), pipettor, 100 to 1000 µL (VWR Catalog #4000-208, Rochester, 10 NY), pipettor, 2 to 20 µL (VWR Catalog #4000-202, Rochester, NY), pipettor, 20 to 200 µL (VWR Catalog #4000-204, Rochester, NY), plate reader (Bio-tek Instruments Model #Elx800, Winooski, VT), PURELAB Plus Water Polishing System (U.S. Filter, Lowell, MA), refrigerator, 4°C (Forma Model #F3775, Marietta, Ohio).

15 *Chemicals, Reagents and Buffers* - Prostaglandin E₂ EIA Kit-Monoclonal 480-well (Cayman Chemical Catalog # 514010, Ann Arbor, MI), centrifuge tube, 50 mL, conical, sterile (VWR Catalog #20171-178, Rochester, NY), Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech Catalog #10-013-CV, Herndon, VA), graduated cylinder, 100 mL (VWR Catalog #24711-20 310, Rochester, NY), KimWipes (Kimberly Clark Catalog #34256, Roswell, GA), microfuge tubes, 1.7 mL (VWR Catalog #20172-698, Rochester, NY), penicillin/streptomycin (Mediatech Catalog #30-001-CI, Herndon, VA), pipet tips for 0.5 to 10 µL pipettor (VWR Catalog #53509-138, Rochester, NY), pipet tips for 100-1000 µL pipettor (VWR Catalog #53512-294, Rochester, NY), 25 pipet tips for 2-20 µL and 20-200 µL pipettors (VWR Catalog #53512-260, Rochester, NY), pipets, 25 mL (Becton Dickinson Catalog #7551, Marietta, OH), storage bottle, 100 mL (Corning Catalog #1395-100, Corning, NY), storage bottle, 1000 mL (Corning Catalog #1395-1L, Corning, NY), ultra-pure water (Resistance =18 megaOhm-cm deionized water).

30 *General Procedure* - EIA Buffer was prepared by diluting the contents of the EIA Buffer Concentrate (vial #4) with 90ml of Ultra-pure water. Vial #4

was rinsed several times to ensure all crystals had been removed and was then placed into a 100 mL storage bottle and stored at 4°C.

The Wash Buffer was prepared by diluting Wash Buffer Concentrate (vial #5) 1:400 with Ultra-pure water. 0.5 ml/liter of Tween 20 (vial #5a) was 5 then added (using a syringe for accurate measurement). To prepare one liter of Wash Buffer add 2.5ml Wash Buffer Concentrate, 0.5ml Tween-20, and 997ml Ultra-pure water. The solution was stored in a 1 liter storage bottle at 4°C.

The Prostaglandin E₂ standard was reconstituted as follows. A 200µL pipet tip was equilibrated by repeatedly filling and expelling the tip several 10 times in ethanol. The tip was used to transfer 100 µL of the PGE₂ Standard (vial #3) into a 1.7 mL microfuge tube. 900µl Ultra-pure water was added to the tube and stored at 4°C, which was stable for ~6 weeks. The Prostaglandin E₂ acetylcholinesterase tracer was reconstituted as follows. 100 µL PGE₂ tracer 15 (vial #2) was mixed with 30 mL of the EIA Buffer in a 50 mL centrifuge tube and stored at 4°C.

The Prostaglandin E₂ monoclonal antibody was reconstituted as follows. 100µL PGE₂ Antibody (vial #1) was mixed with 30 mL of the EIA buffer in a 50 mL centrifuge tube and stored at 4°C.

DMEM with penicillin/streptomycin was prepared by adding 5 mL 20 penicillin/streptomycin into 500 mL DMEM and stored at 4°C.

The plates were set up as follows: Each plate contained a minimum of two blanks (B), two non-specific binding wells (NSB), two maximum binding wells (B₀), and an eight point standard curve run in duplicate (S1-S8). Each sample was assayed at a minimum of two dilutions and each dilution was run in 25 duplicate.

The standard was prepared as follows: Eight 1.7 mL microuge tubes 30 were labeled as tubes 1-8. 900 µL DMEM into was added to tube 1 and 500 µL DMEM to tubes 2-8. 100 µL of the PGE₂ standard was added to tube 1 and mixed. Five-hundred mL of solution was taken from tube 1 and put into tube 2, and this process was repeated through tube 8.

Fifty mL EIA Buffer and 50 μ L DMEM were added into the NSB wells. Fifty μ L DMEM was added to the B₀ wells. Fifty mL of solution was taken from tube #8 and added to both the lowest standard wells (S8). Fifty mL was taken from tube #7 and added to each of the next two wells. This was continued through to tube #1. (the same pipet tip was used for all 8 of the standards making sure to equilibrate the tip in each new standard by pipeting up and down in that standard. Using a P200, 50 μ L of each sample at each dilution was added to the sample wells).

Using a 12 channel pipetor, 50 μ L of the Prostaglandin E₂ acetylcholinesterase tracer was added to each well except the Total Activity (TA) and the Blank (B) wells. Using the 12 channel pipetor, 50 μ L of the Prostaglandin E₂ monoclonal antibody was added to each well except the Total Activity (TA), the (NSB), and the Blank (B) wells. The plate was covered with plastic film (item #7) and incubated for 18 hours at 4°C.

The plates were developed as follows: one 100 μ L vial of Ellman's Reagent (vial #8) was reconstituted with 50 ml of Ultra-pure water in a 50 mL centrifuge tube. It was protected from light and used the same day. The wells were washed and rinsed five times with Wash Buffer using a 12 channel pipettor. Two-hundred mL of Ellman's Reagent was added to each well using a 12 channel pipettor and 5 μ L of Tracer to the total activity(TA) wells was then added to each well using a P10 pipette. The plate was covered with a plastic film and placed on orbital shaker in the dark for 60-90 minutes.

The plate was read in the Bio-tek plate reader at a single wavelength between 405 and 420 nm. Before reading each plate, the bottom was wiped with a Kim wipe. The plate should be read when the absorbance of the wells is in the range of 0.3-0.8 A.U. If the absorbance of the wells exceeded 1.5, they were washed and fresh Ellmans' Reagent was added and then redeveloped.

Calculation of synergy and combination index

Synergy between parthenolide and andrographolide was assessed using CalcuSyn (BIOSOFT, biosoft.com). This statistical package performs multiple

drug dose-effect calculations using the Median Effect methods described by T-C Chou and P. Talaly (Trends Pharmacol. Sci. 4:450-454), hereby incorporated by reference.

Briefly, it correlates the "Dose" and the "Effect" in the simplest possible form: $fa/fu = (C/C_m)^m$, where C is the concentration or dose of the compound and C_m is the median-effective dose signifying the potency. C_m is determined from the x-intercept of the median-effect plot. The fraction affected by the concentration of the test material is fa and the fraction unaffected by the concentration is fu ($fu = 1 - fa$). The exponent m is the parameter signifying the sigmoidicity or shape of the dose-effect curve. It is estimated by the slope of the median-effect plot.

The median-effect plot is a plot of $x = \log(C)$ vs $y = \log(fa/fu)$ and is based on the logarithmic form of Chou's median-effect equation. The goodness of fit for the data to the median-effect equation is represented by the linear correlation coefficient r of the median-effect plot. Usually, the experimental data from enzyme or receptor systems have an $r > 0.96$, from tissue culture an $r > 0.90$ and from animal systems an $r > 0.85$.

Synergy of test components is quantified using the combination index (CI) parameter. The CI of Chou-Talaly is based on the multiple drug-effect and is derived from enzyme kinetic models (Chou, T.-C. and Talalay, P. (1977) A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. J. Biol. Chem. 252:6438-6442). The equation determines only the additive effect rather than synergism or antagonism. However, synergism is defined as a more than expected additive effect, and antagonism as a less than expected additive effect as proposed by Cho and Talalay in 1983 (Trends Pharmacol. Sci. (1983) 4:450-454). Using the designation of $CI = 1$ as the additive effect, there is obtained for mutually exclusive compounds that have the same mode of action or for mutually non-exclusive drugs that have totally independent modes of action the following relationships: $CI < 1$, $= 1$, and > 1 indicating synergism, additivity and antagonism, respectively.

Expected median inhibitory concentrations of the two-component combinations were estimated using the relationship:

$$[1/\text{Expected IC}_{50}] = [A/\text{IC}_{50}\text{A}] + [B/\text{IC}_{50}\text{B}]$$

where A = mole fraction of component A in the combination and B = the mole fraction of component B in the combination.

TABLE 3 illustrates the observed and expected median inhibitory concentrations for parthenolide and andrographolide for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected IC₅₀ for the 1:10 combination of parthenolide and andrographolide was 4.25 µg/mL, the observed value was 2.2 µg/mL or 2.8-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the 1:10 combination of parthenolide and andrographolide.

15 TABLE 3. Observed and Expected Median Inhibitory Concentrations for a (10:1) Formulation of parthenolide and andrographolide

Components (1:10)	Parthenolide IC ₅₀ (µg/mL)	Andrographolide IC ₅₀ (µg/mL)	Combination	
			Expected IC ₅₀ (µg/mL)	Observed IC ₅₀ (µg/mL)
Parthenolide: Andrographolide	0.56	12.2	4.25	2.18

20 Statistical analysis of inhibition of COX-2 production of PGE2 in the RAW 264.7 cell model for the 1:10 combination of parthenolide and is presented in TABLE 4. The CI for this combination was 0.359, 0.969 and 2.65, respectively, for the IC₅₀, IC₇₅ and IC₉₀. These CI values indicate strong synergy between parthenolide and andrographolide over the complete dose-response curve.

30 TABLE 4. Combination Index for a 1:10 Formulation of parthenolide and andrographolide.

Combination Index			Mean CI
IC50	IC75	IC90	
0.359	0.969	2.65	1.33

These data are with and support the test results and conclusions performed in the Jurkat cells in which COX-2 protein expression was monitored.

5

EXAMPLE 16

Inhibition of COX-2 Enzyme Production of Prostaglandin E2 in Murine B Cells by Parthenolide and Oleanolic Acid

This example illustrates the superior COX-2 inhibitory potency and selectivity of the combination of parthenolide and oleanolic acid of the present invention compared to parthenolide or oleanolic acid alone. The experiments were performed as described in EXAMPLE 15 with parthenolide and oleanolic acid in stead of parthenolide and andrographolide.

TABLE 5 illustrates the observed and expected median inhibitory concentrations for parthenolide and oleanolic acid for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected IC₅₀ for the 1:4 combination of parthenolide and oleanolic acid was 2.8 µg/mL, the observed value was 0.67 µg/mL or 4.2-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the 1:4 combination of parthenolide and oleanolic acid.

TABLE 5. Observed and Expected Median Inhibitory Concentrations for a Formulation of Parthenolide and Oleanolic acid.

Components (1:4)	Parthenolide IC ₅₀ (µg/mL)	Oleanolate IC ₅₀ (µg/mL)	Combination	
			Expected IC ₅₀ (µg/mL)	Observed IC ₅₀ (µg/mL)
Parthenolide: Oleanolic Acid	0.56	9.5	2.3	0.67

Statistical analysis of inhibition of COX-2 production of PGE2 in the RAW 264.7 cell model for the 1:4 combination of parthenolide and oleanolic acid is presented in TABLE 6. The CI for this combination was 0.552, 0.890 and 1.44, respectively, for the IC₅₀, IC₇₅ and IC₉₀. These CI values indicate

strong synergy between parthenolide and oleanolic acid over the complete dose-response curve.

TABLE 6. Combination Index for a 1:10 Formulation of Parthenolide and Oleanolic Acid

5	Combination Index			Mean CI
	IC50	IC75	IC90	
	0.552	0.890	1.44	

These data are with and support the test results and conclusions performed in the Jurkat cells in which COX-2 protein expression was monitored.

EXAMPLE 17

Inhibition of COX-2 Enzyme Production of Prostaglandin E2 in Murine Cells by Parthenolide and Ursolic Acid

This example illustrates the superior COX-2 inhibitory potency and selectivity of the combination of parthenolide and ursolic acid of the present invention compared to parthenolide or ursolic acid alone. The experiments were performed as described in EXAMPLE 15 with parthenolide and ursolic acid instead of parthenolide and andrographolide.

TABLE 7 illustrates the observed and expected median inhibitory concentrations for parthenolide and ursolic acid for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected IC₅₀ for the 1:4 combination of parthenolide and ursolic acid was 2.5 µg/mL, the observed value was 0.56 µg/mL or 4.5-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the 1:4 combination of parthenolide and ursolic acid.

TABLE 7. Observed and Expected Median Inhibitory Concentrations for a Formulation of Parthenolide and Ursolic Acid

30	Components (1:4)	Combination		
		Parthenolide IC ₅₀ (µg/mL)	Ursolate IC ₅₀ (µg/mL)	Expected IC ₅₀ (µg/mL)

Parthenolide: Ursolic Acid	0.56	16.1	2.5	0.56
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Statistical analysis of inhibition of COX-2 production of PGE2 in the
 5 RAW 264.7 cell model for the 1:4 combination of parthenolide and ursolic acid
 is presented in TABLE 6. The CI for this combination was 0.307 0.306 and
 0.451, respectively, for the IC₅₀, IC₇₅ and IC₉₀. These CI values indicate strong
 synergy between parthenolide and ursolic acid over the complete dose-response
 curve.

10

TABLE 8. Combination Index for a 1:10 Formulation of Parthenolide and Ursolic Acid.

15	Combination Index			Mean CI
	IC50	IC75	IC90	
	0.307	0.369	0.451	0.376

These data are with and support the test results and conclusions
 performed in the Jurkat cells in which COX-2 protein expression was
 20 monitored.

EXAMPLE 18

Inhibition of COX-2 Enzyme Production of Prostaglandin E2 in Murine B Cells by Parthenolide and Triptolide

25 This example illustrates the superior COX-2 inhibitory potency and selectivity of the combination of parthenolide and triptolide of the present invention compared to parthenolide or triptolide alone. The experiments were performed as described in EXAMPLE 15 with parthenolide and triptolide instead of parthenolide and andrographolide.

30 TABLE 9 illustrates the observed and expected median inhibitory concentrations for parthenolide and triptolide for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected IC₅₀ for the 1:2 combination of parthenolide and triptolide was 0.131 µg/mL, the observed value was 0.032

$\mu\text{g/mL}$ or 4.1-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the 1:10 combination of parthenolide and triptolide.

5

Components (1:2)	Combination			
	Parthenolide $\text{IC}_{50}(\mu\text{g/mL})$	Triptolide $\text{IC}_{50}(\mu\text{g/mL})$	Expected $\text{IC}_{50}(\mu\text{g/mL})$	Observed $\text{IC}_{50}(\mu\text{g/mL})$
Parthenolide: Triptolide	0.560	0.094	0.131	0.032

10 Statistical analysis of inhibition of COX-2 production of PGE2 in the RAW 264.7 cell model for the 1:2 combination of parthenolide and triptolide is presented in TABLE 10. The CI for this combination was 0.250, 0.259 and 0.349, respectively, for the IC_{50} , IC_{75} and IC_{90} . These CI values indicate strong synergy between parthenolide and triptolide over the complete dose-response curve.

15

TABLE 10. Combination Index for a 1:10 Formulation of Parthenolide and Triptolide.

20

Combination Index			Mean CI
IC_{50}	IC_{75}	IC_{90}	
0.250	0.295	0.349	0.298

25

These date are with and support the test results and conclusions performed in the Jurkat cells in which COX-2 protein expression was monitored.

EXAMPLE 19

Inhibition of COX-2 Enzyme Production of Prostaglandin E2
30 in Murine B Cells by Triptolide and Andrographolide

This example illustrates the superior COX-2 inhibitory potency and selectivity of the combination of triptolide and andrographolide of the present

invention compared to triptolide or andrographolide. The experiments were performed as described in EXAMPLE 15 with triptolide and andrographolide instead of parthenolide and andrographolide.

5 TABLE 11 describes the observed and expected median inhibitory concentrations for triptolide and andrographolide for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected IC₅₀ for the 1:4 combination of triptolide:andrographolide was 0.469 µg/mL, the observed value was 0.260 µg/mL or 1.8-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the
10 1:4 combination of triptolide and andrographolide.

TABLE 11. Observed and Expected Median Inhibitory Concentrations for a Formulation of Triptolide and Andrographolide.

15

Components (1:4)	Triptolide IC ₅₀ (µg/mL)	Andrographolide IC ₅₀ (µg/mL)	Combination	
			Expected IC ₅₀ (µg/mL)	Observed IC ₅₀ (µg/mL)
Triptolide: Andrographolide	0.094	12.2	0.469	0.260

20 Statistical analysis of inhibition of COX-2 production of PGE2 in the RAW 264.7 cell model for the 1:4 combination of triptolide and andrographolide is presented in TABLE 12. The CI for this combination was 0.551, 0.546 and 0.542, respectively, for the IC₅₀, IC₇₅ and IC₉₀. These CI
25 values indicate strong synergy between triptolide and andrographolide, while the mean CI value of 0.546 indicates strong synergy over the entire dose-response range.

30 TABLE 12. Combination Index for a 1:4 Formulation of Triptolide and Andrographolide.

Combination Index			Mean CI
IC50	IC75	IC90	
0.551	0.546	0.542	0.546

These data are with and support the test results and conclusions performed in the Jurkat cells in which COX-2 protein expression was monitored.

5

EXAMPLE 20

Inhibition of COX-2 Enzyme Production of Prostaglandin E2 in Murine B Cells by Triptolide and Oleanolic Acid

This example illustrates the superior COX-2 inhibitory potency and selectivity of the combination of triptolide and oleanolic acid of the present invention compared to triptolide or oleanolic acid alone. The experiments were performed as described in EXAMPLE 15 with triptolide and oleanolic acid instead of parthenolide and andrographolide.

TABLE 13 describes the observed and expected median inhibitory concentrations for triptolide and oleanolic acid for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected IC₅₀ for the 1:4 combination of triptolide:oleanolic acid was 1.03 µg/mL, the observed value was 0.67 µg/mL or 1.6-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the 1:4 combination of triptolide and oleanolic acid.

20

TABLE 13. Observed and Expected Median Inhibitory Concentrations for a Formulation of Triptolide and Oleanic Acid

25

Components (1:4)	Combination			
	Triptolide IC ₅₀ (µg/mL)	Oleanolate IC ₅₀ (µg/mL)	Expected IC ₅₀ (µg/mL)	Observed IC ₅₀ (µg/mL)
Triptolide: Oleanolic Acid	0.094	9.50	1.039	0.667

30

Statistical analysis of inhibition of COX-2 production of PGE2 in the RAW 264.7 cell model for the 1:4 combination of triptolide and oleanolic acid is presented in TABLE 14. The CI for this combination was 0.642, 0.562 and 0.493, respectively, for the IC₅₀, IC₇₅ and IC₉₀. These CI values indicate strong

50

synergy between triptolide and oleanolic acid, while the mean CI value of 0.566 indicates strong synergy over the entire dose-response range.

5 TABLE 14. Combination Index for a 1:4 Formulation of Triptolide and
Oleanolic Acid

	Combination Index			Mean CI
	IC50	IC75	IC90	
	10 0.642	0.562	0.493	

15 These data are with and support the test results and conclusions performed in the Jurkat cells in which COX-2 protein expression was monitored.

15

EXAMPLE 21

Inhibition of COX-2 Enzyme Production of Prostaglandin E2 in Murine B Cells by Triptolide and Ursolic Acid

20 This example illustrates the superior COX-2 inhibitory potency and selectivity of the combination of triptolide and ursolic acid of the present invention compared to triptolide or ursolic acid alone. The experiments were performed as described in EXAMPLE 15 with triptolide and ursolic acid instead of parthenolide and andrographolide.

25 TABLE 15 describes the observed and expected median inhibitory concentrations for triptolide and ursolic acid for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected IC_{50} for the 1:4 combination of triptolide:ursolic acid was 1.03 μ g/mL, the observed value was 0.67 μ g/mL or 1.6-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the 1:4
30 combination of triptolide and ursolic acid.

Components (1:4)	Combination			
	Triptolide IC ₅₀ (μg/mL)	Ursolate IC ₅₀ (μg/mL)	Expected IC ₅₀ (μg/mL)	Observed IC ₅₀ (μg/mL)
5 Triptolide: Ursolic Acid	0.094	16.1	0.486	0.240

Statistical analysis of inhibition of COX-2 production of PGE2 in the RAW 264.7 cell model for the 1:4 combination of triptolide and ursolic acid is presented in TABLE 16. The CI for this combination was 0.511, 0.523 and 0.537, respectively, for the IC₅₀, IC₇₅ and IC₉₀. These CI values indicate strong synergy between triptolide and ursolic acid, while the mean CI value of 0.524 indicates strong synergy over the entire dose-response range.

15 TABLE 16. Combination Index for a 1:4 Formulation of Triptolide and Ursolic Acid.

Combination Index			Mean CI
IC50	IC75	IC90	
20 0.511	0.523	0.537	0.524

These data are with and support the test results and conclusions performed in the Jurkat cells in which COX-2 protein expression was monitored.

Thus, among the various formulations taught there has been disclosed a formulation comprising triptolide or parthenolide, as the first component, and a compound selected from the group consisting of triptolide, parthenolide, andrographolide, ursolic acid and oleanolic acid, as the second component. These combinations provide for a synergistic anti-inflammatory effect in response to physical or chemical injury or abnormal immune stimulation due to a biological agent or unknown etiology. It will be readily apparent to those skilled in the art that various changes and modifications of an obvious nature may be made without departing from the spirit of the invention, and all such

changes and modifications are considered to fall within the scope of the invention as defined by the appended claims. Such changes and modifications would include, but not be limited to, the incipient ingredients added to affect the capsule, tablet, lotion, food or bar manufacturing process as well as vitamins, 5 herbs, flavorings and carriers. Other such changes or modifications would include the use of other herbs or botanical products containing the combinations of the present invention disclosed above.

CLAIMS

We claim:

1. A composition for inhibition of inducible COX-2 activity and having minimal effect on COX-1 activity, said composition comprising an effective amount of a first component comprising a member selected from the group consisting of a diterpene triepoxide lactone species and a sesquiterpene lactone species and an effective amount of a second component selected from the group consisting of a diterpene triepoxide lactone species, a sesquiterpene lactone species, a diterpene lactone species, and a triterpene species or derivatives thereof with the proviso that the same diterpene triepoxide lactone species or sesquiterpene lactone species cannot concurrently serve as both said first and second component.
10
2. The composition of Claim 1 wherein the diterpene triepoxide lactone species is of pharmaceutical grade and is a member selected from the group consisting of triptolide, triptonide, tripdiolide and tripchlorolide.
15
3. The composition of Claim 1 wherein the sesquiterpene lactone species is of pharmaceutical grade and is a member selected from the group consisting of parthenolide, encelin, leucanthin B, enhydrin, melapodin A, tenulin, confertiflorin, burrodin, psilostachyin A, costunolide, strigol, helenalin, 5-a-hydroxy-dehydrocostuslactone, chlorochrymorin, chrysandiol, chrysartemin A, chrysartemin B, cinerenin, curcolone, cynaropicrin, dehydrocostus lactone, dehydroleucodin, dehydrozaluzanin C, deoxylatucin, eremanthe,
20
- 25
25. eupaformonin, eupaformosanin, eupatolide, furanodienone, heterogorgiolide, lactucin, magnolialide, michelenolide, repin, spirafolide, and zaluzanin C.
4. The composition of Claim 1 wherein the diterpene lactone species is of pharmaceutical grade and is a member selected from the group consisting of andrographolide, dehydroandrographolide, deoxyandrographolide, neoandrographolide, selenoandrographolide, homoandrographolide,
30

andrographan, amdrographon, andrographosterin, 14-deoxy-11-oxoandrographolide, 14-deoxy-11, 12-didehydroandrographolide, andrographiside, and edelin lactone.

- 5 5. The composition of Claim 1 wherein the triterpene species is of pharmaceutical grade and is a member selected from the group consisting of ursolic acid, oleanolic acid, betulin, betulinic acid, glycyrrhetic acid, glycyrrhizic acid, triperin, 2- α -3- α -dihydroxyurs-12-3n-28-oic acid, 2- α -hydroxyursolic acid, 3-oxo-ursolic acid, celastrol, friedelin, tritophenolide, 10 uvaol, eburicoic acid, glycyrrhizin, gypsogenin, oleanolic acid-3-acetate, pachymic acid, pinolic acid, sophoradiol, soyasapogenol A, soyasapogenol B, tumulosic acid, ursolic acid-3-acetate and sitosterol.
- 15 6. The composition of Claim 1 wherein first and second components are derived from plants or plant extracts.
- 20 7. The composition of Claim 1 wherein at least one of said first or second components is conjugated with a compound selected from the group consisting of mono- or di- saccharides, amino acids, sulfates, succinate, acetate and glutathione.
- 25 8. The composition of Claim 1, formulated in a pharmaceutically acceptable carrier.
- 30 9. The composition of Claim 1, additionally containing one or more members selected from the group consisting of antioxidants, vitamins, minerals, proteins, fats, carbohydrates, glucosamine, chondroitin sulfate and aminosugars.
10. A method of dietary supplementation in animals comprising administering to an animal suffering symptoms of inflammation a composition

of one of the Claims 1 to 9 and continuing said administration until said symptoms are reduced.

5 11. The method of Claim 10 wherein the composition is formulated in a dosage form such that said administration provides from 0.001 to 3.0 mg body weight per day of each diterpene triepoxide lactone species, from 0.05 to 5.0 mg body weight per day of each sequesterpene lactone species and from 0.5 to 20.0 mg/kg body weight per day of each diterpene lactone or triterpene species.

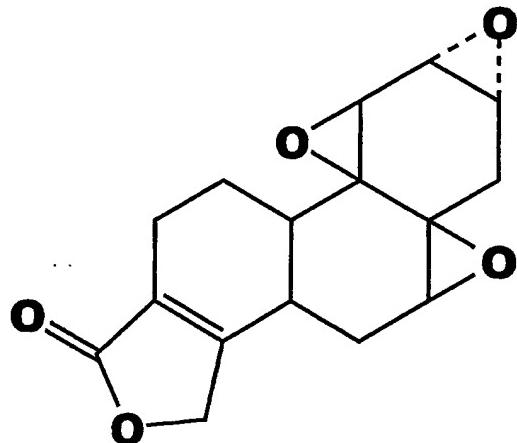
10 12. The method of Claim 10, wherein the composition is administered in an amount sufficient to maintain a serum concentration of 0.1 to 10 nM of each diterpene triepoxide lactone species; from 0.001 to 10 μ M of each sequesterpene lactone species, and from 0.001 to 10 μ M of each diterpene lactone or triterpene species.

15 13. A method of therapeutic treatment in animals comprising administering to an animal suffering symptoms of arthritis a composition of one of the Claims 1 to 9 and continuing said administration until said symptoms are reduced.

20 14. A method of therapeutic treatment comprising applying to the skin of a human suffering symptoms of acne rosacea a lotion comprising a composition of one of the Claims 1 to 9 and continuing said administration until said symptoms are reduced.

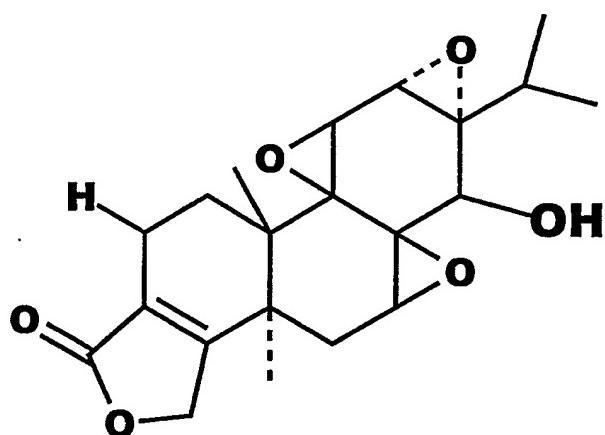
25 15. A method of therapeutic treatment comprising applying to the skin of a human suffering symptoms of psoriasis a lotion comprising a composition of one of the Claims 1 to 9 and continuing said administration until said symptoms are reduced.

[A1]



Diterpene Triepoxide Lactone

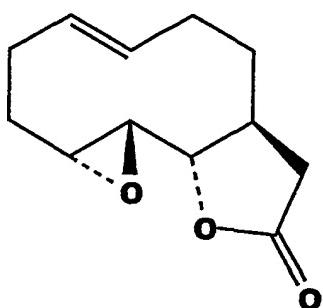
[A2]



Triptolide

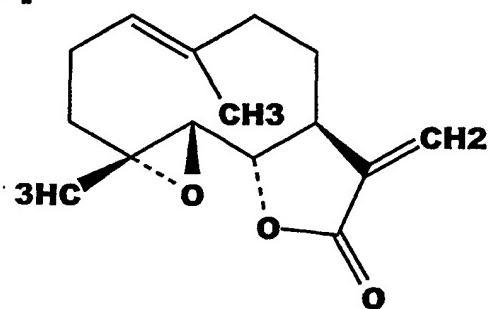
Fig. 1

[A1]



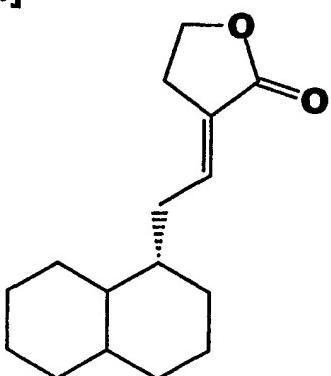
Sesquiterpene lactone

[A2]



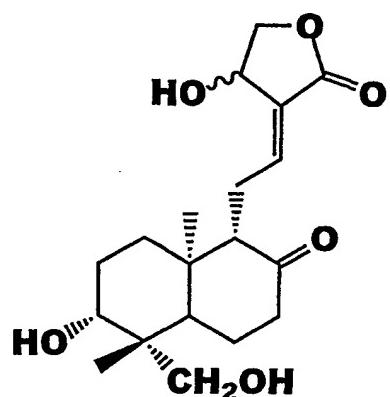
Parthenolide

[B1]



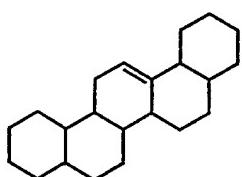
Diterpene Lactone

[B2]



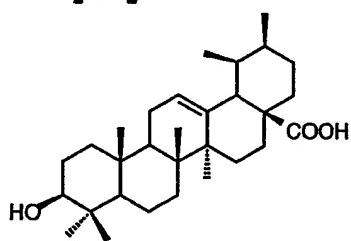
Andrographolide

[C1]



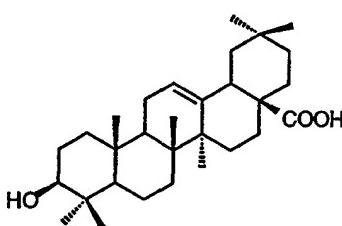
Triterpene

[C2]



Ursolic Acid

[C3]



Oleanolic Acid

Fig. 2

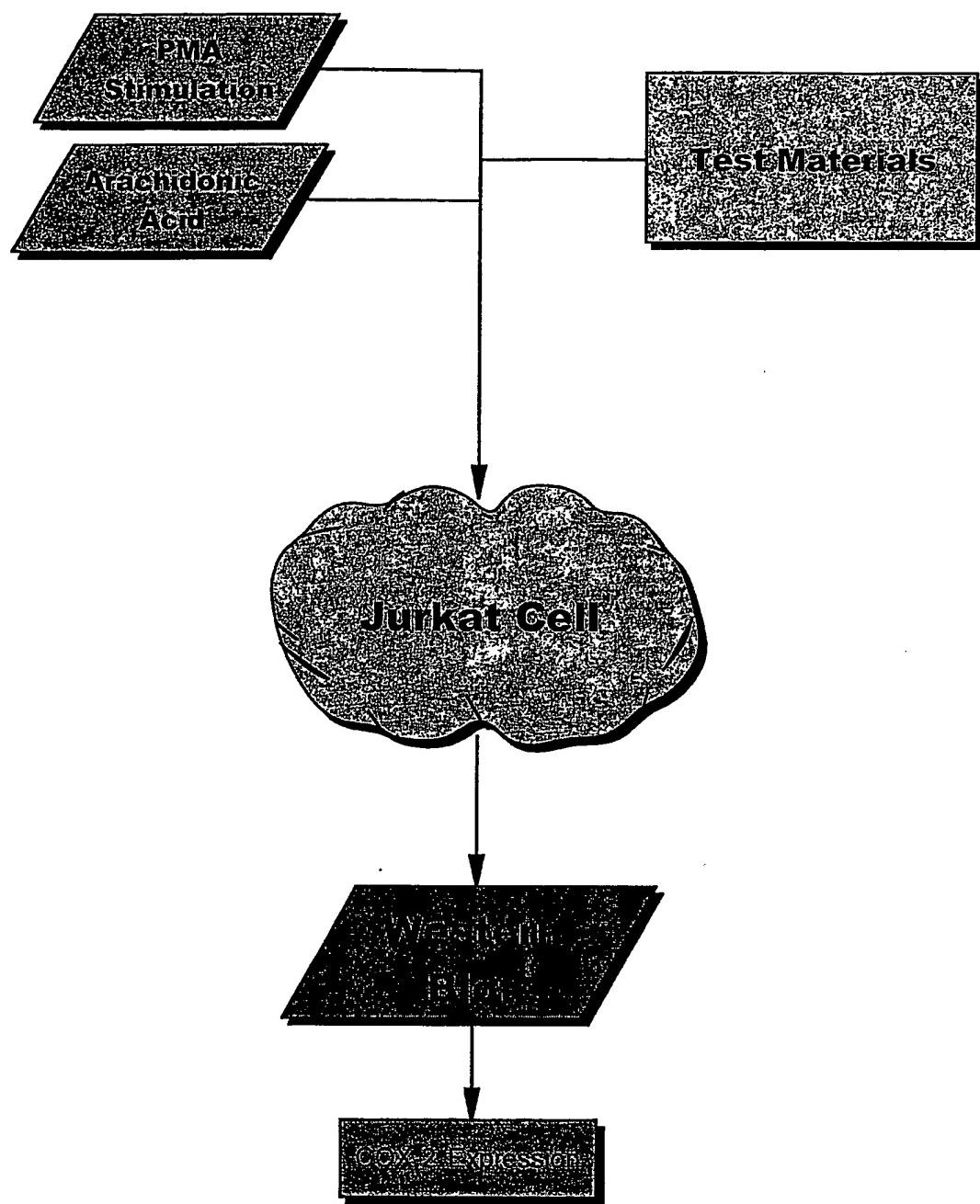


Fig. 3

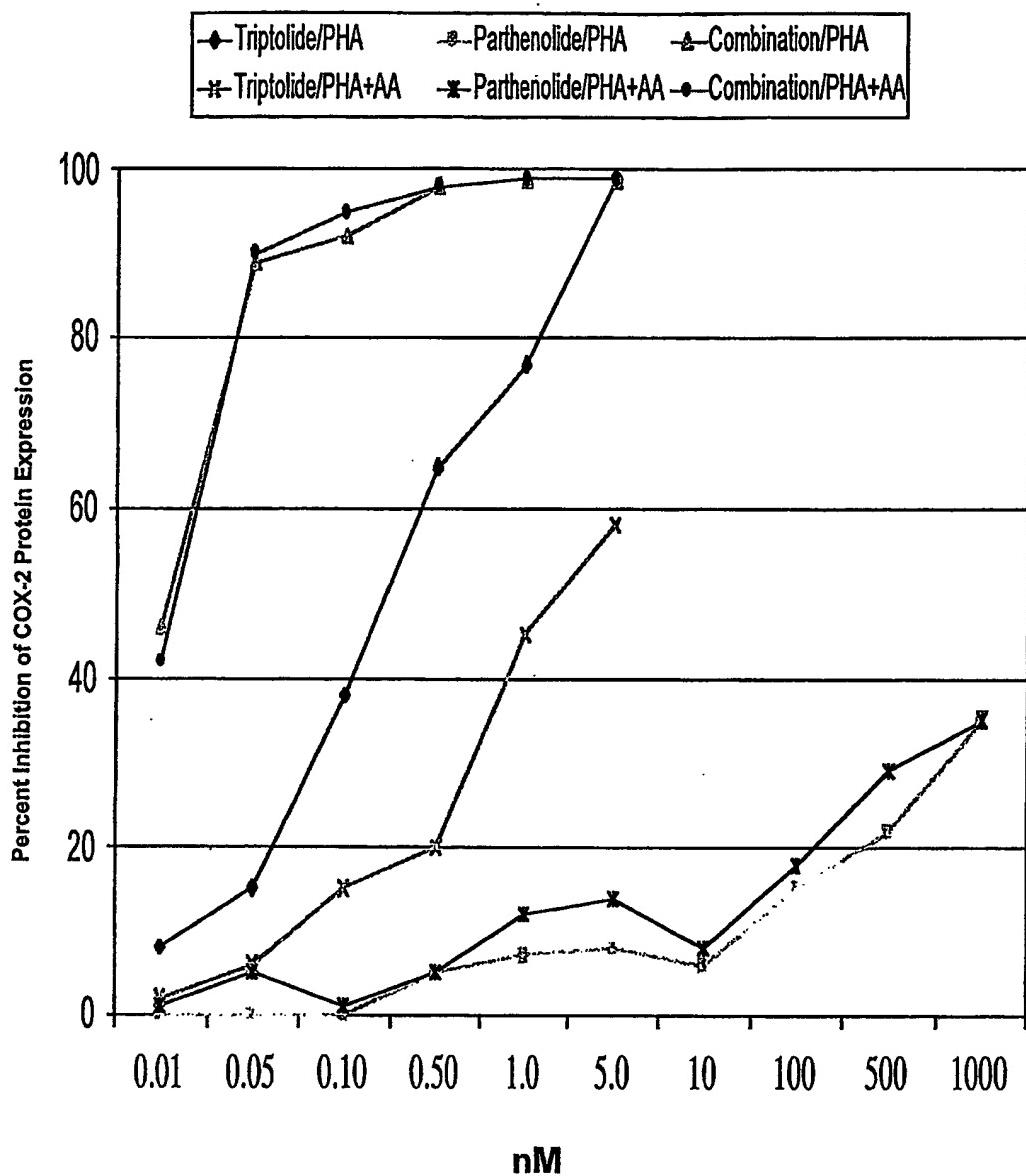


Fig. 4 (a)

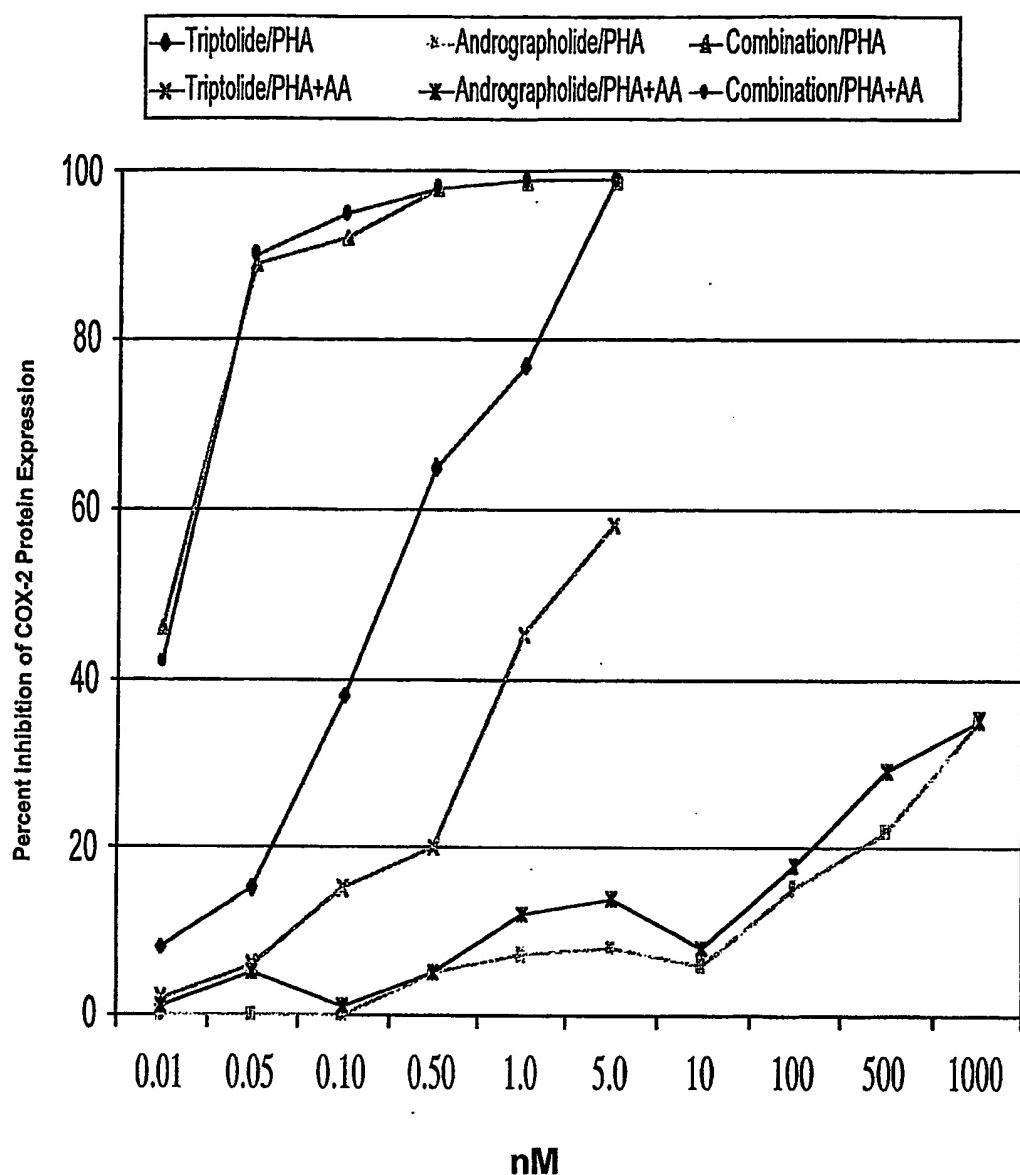


Fig. 4 (b)

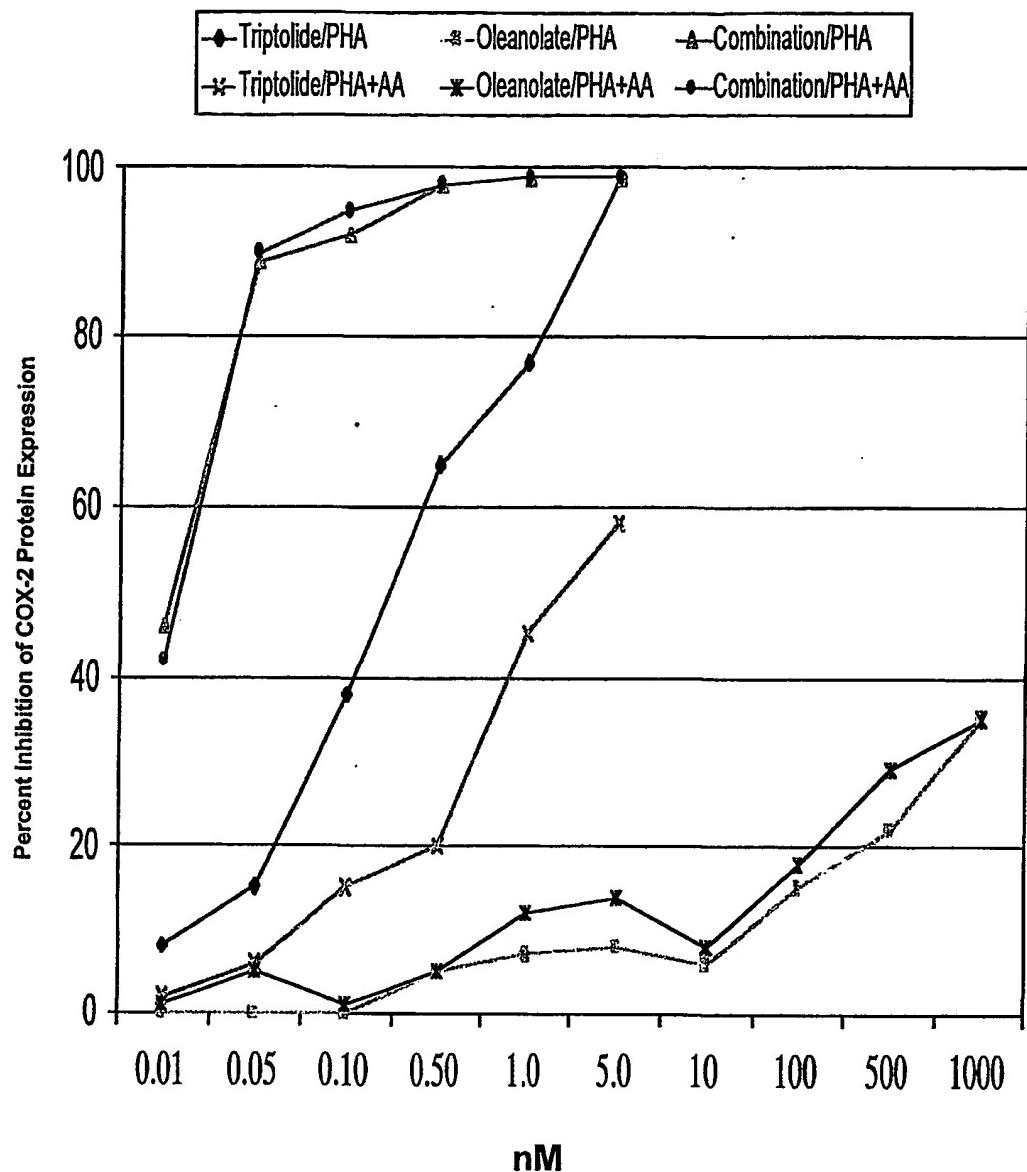


Fig. 4 (c)

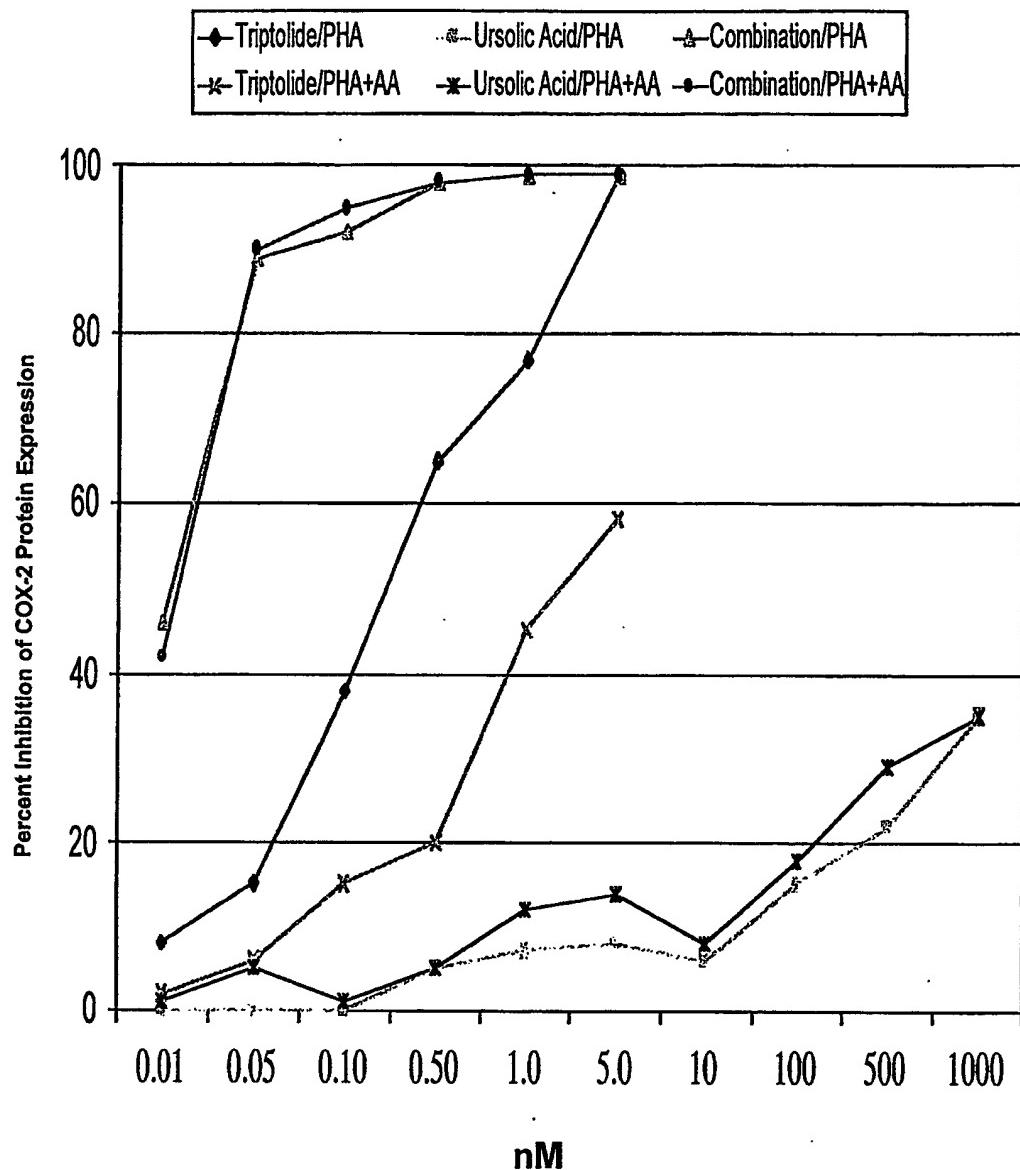


Fig. 4 (d)

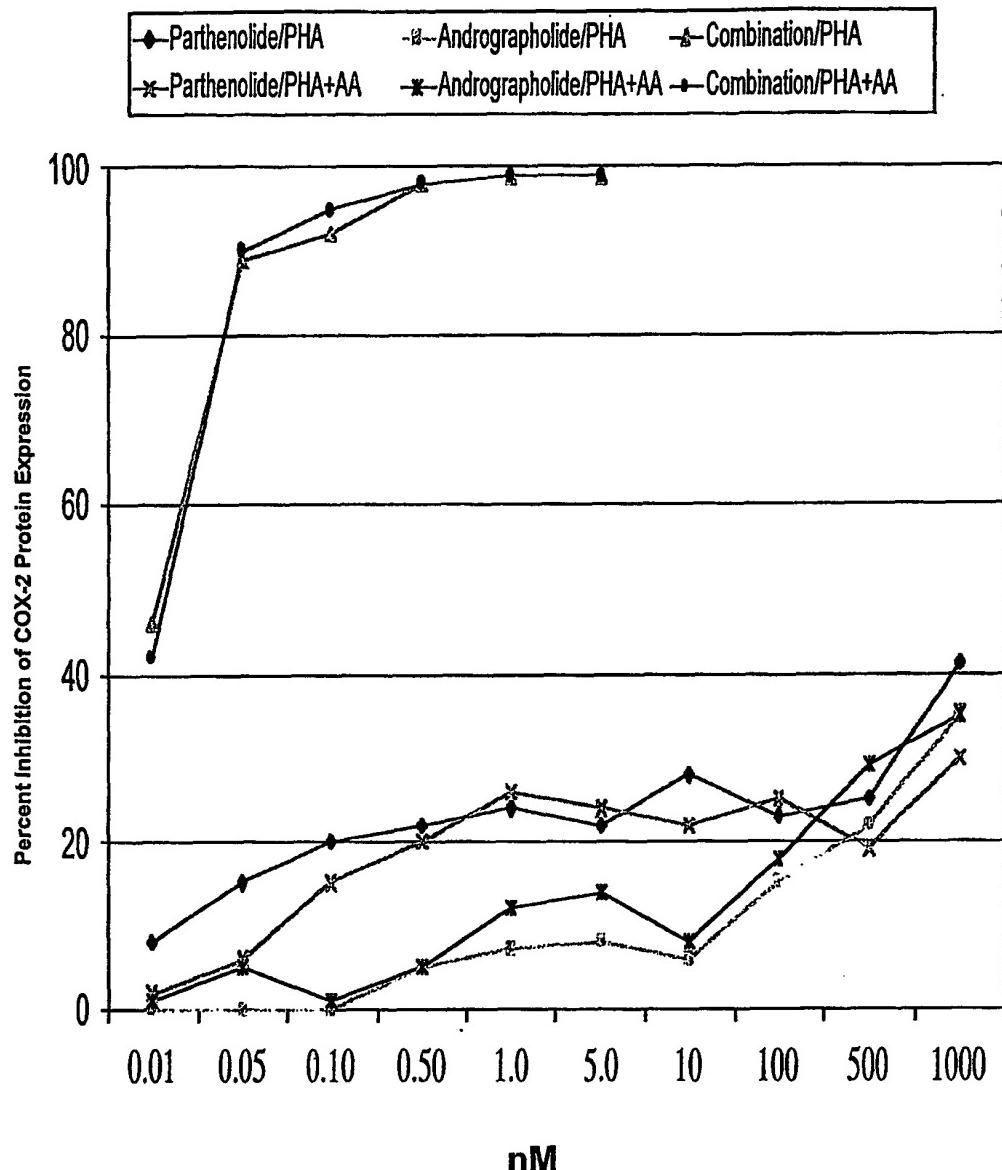


Fig. 4 (e)

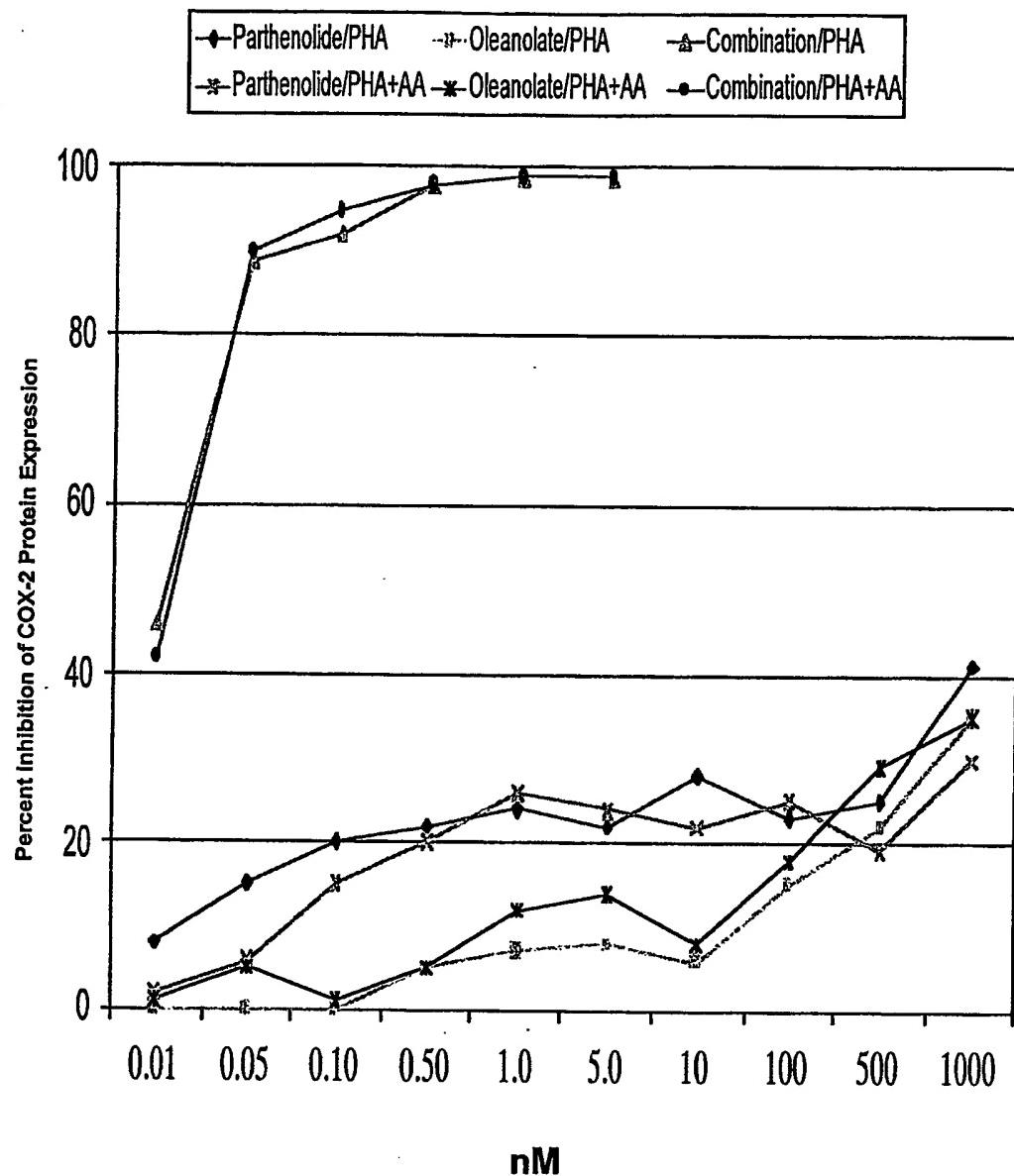


Fig. 4 (f)

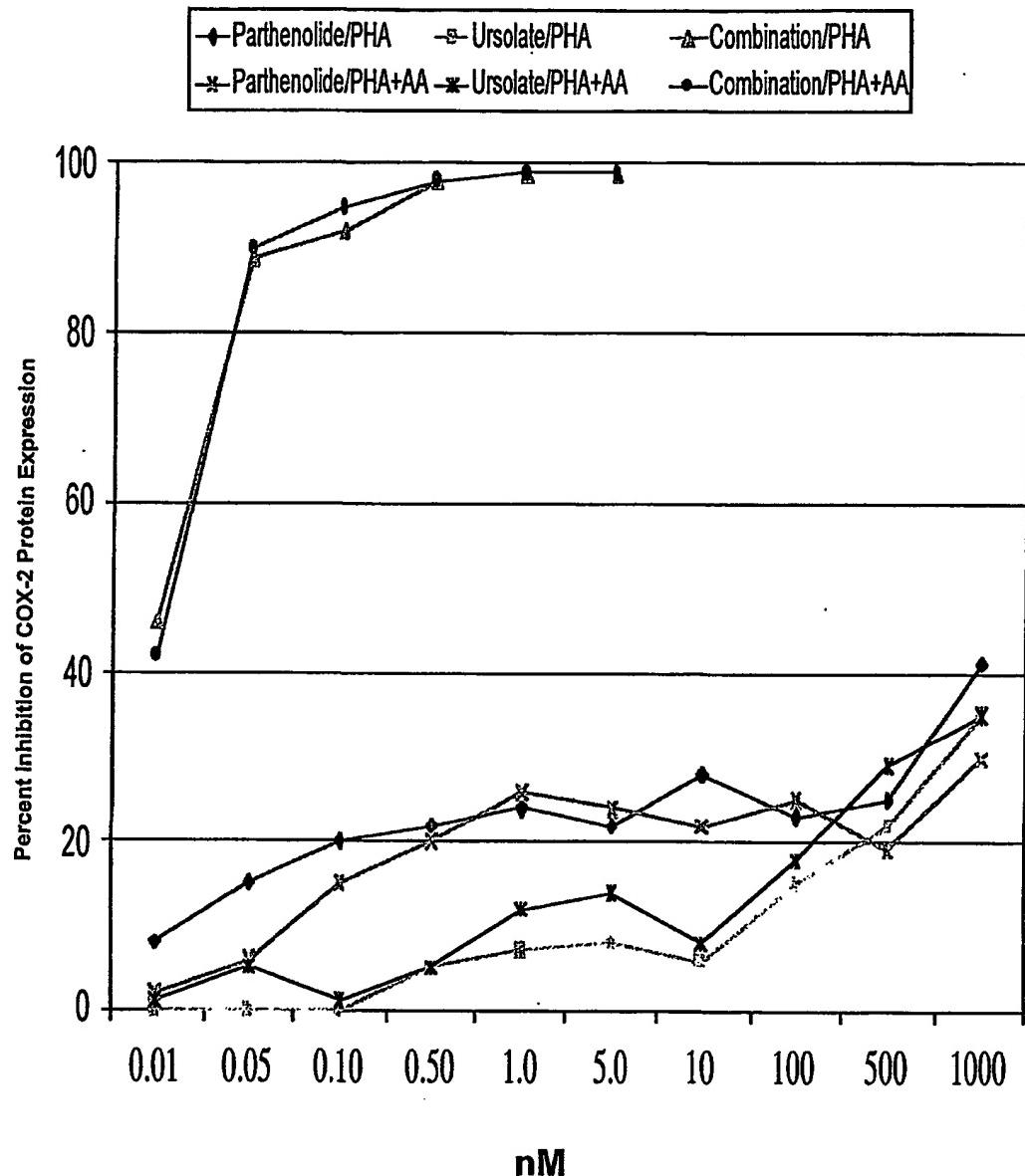


Fig. 4 (g)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/24053

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/34

US CL : 514/468

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/468

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,962,516 A (QI ET AL) 05 October 1999 (05.10.99), see entire document.	1-15

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance		
"E" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O" document referring to an oral disclosure, use, exhibition or other means	"G"	document member of the same patent family
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Date of the actual completion of the international search	Date of mailing of the international search report
16 NOVEMBER 2001	15 JAN 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer <i>Janie Ford</i> FREDERICK KRASS Telephone No. (703) 305-1235
Facsimile No. (703) 305-3230	